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John P. Neoptolemos
Raul Urrutia
James L. Abbruzzese
Markus W. Büchler *Editors*

Pancreatic Cancer

Second Edition

Springer

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With 244 Figures and 93 Tables

Springer

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This work is dedicated to our wives Linda, Gwen, Marie, and Hedi and to our patients and their relatives

Foreword

We have seen remarkable advances in the understanding of the epidemiologic, cellular, and molecular pathogenesis of pancreatic cancer since the First Edition of *Pancreatic Cancer* in 2010. Yet pancreatic cancer is still one of the most common causes of cancer-related death among men and women in the USA and many other parts of the world, and the incidence is increasing relentlessly. The more we seem to know, the bigger the challenge becomes. We said in 2010 "how do we get to the next level?" That next level has now arrived.

In 2010, we were just introduced to FOLFIRINOX, a highly active chemotherapy regimen. That was soon followed by the finding that gemcitabine and albumin-bound paclitaxel is also very effective. As we have advanced these therapies from metastatic to locally advanced to even resectable/borderline resectable disease, we see the overall survival inching up. A small triumph perhaps, but worth noting. Our challenge now is how to insert more targeted agents and to position immuno-oncology into the arsenal against this very "cold" tumor.

Because of more effective therapy, more and more patients are undergoing resection even in settings where, previously, surgery would not be attempted. Despite the greater complexity of the surgical techniques, the operations themselves remain safe. We are also seeing remarkable advances in laparoscopic and robotic surgeries for selected cases with shorter hospital stay, allowing more patients to be eligible for adjuvant treatment.

Gene testing is now recommended for patients in whom we suspect mutations based on family history, but germline testing for inherited cancer susceptibility seems to be gaining more traction. Recently, researchers at Johns Hopkins found the incidence of these mutations in patients with unremarkable family histories was around 4% and much higher than anyone would have anticipated. They sequenced 32 genes in 854 patients and found 33 with a deleterious germline mutation, including BRCA2 (12 patients), ATM (10 patients), BRCA1 (3 patients), PALB2 (2 patients), MLH1 (2 patients), CDKN2A (1 patient), TP53 (1 patient), BUB1B (1 patient), and BUB3 (1 patient). Some of these susceptibility gene mutations would be missed if current family history guidelines for gene testing were to be applied. Although the proportion of affected individuals was small, the potential treatment impact on these individuals and screening implications for family members are both massive. Patients with germline mutations in DNA repair pathways enjoy exquisite

responses to gemcitabine and cisplatin. PARP inhibitors can benefit patients with BRCA1/2 mutations, and pembrolizumab is effective for patients with microsatellite instability-high (MSI-H) cancer. In addition, we can use knowledge of germline mutations to avoid certain treatments, such as radiation, for those with ATM or TP53 mutations.

The Cancer Genome Atlas Research Network has also just recently performed an integrated genomic, transcriptomic, and proteomic profiling of 150 pancreatic ductal adenocarcinomas. Deep whole exome sequencing revealed recurrent somatic mutations in KRAS, TP53, CDKN2A, SMAD4, RNF43, ARID1A, TGFβR2, GNAS, RREB1, and PBRM1. Interestingly, KRAS wild-type tumors harbored alterations in other oncogenic drivers, including GNAS, BRAF, CTNNB1, and additional RAS pathway genes. A subset of tumors was found to have multiple KRAS mutations, with some showing evidence of biallelic mutations. Going onto protein profiling a favorable prognosis subset was identified with low epithelial-mesenchymal transition and high MTOR pathway activation. Associations of noncoding RNAs with tumor-specific mRNA subtypes were also identified. This is one of the key steps providing a roadmap for precision medicine.

This Second Edition textbook, *Pancreatic Cancer*, has maintained the momentum of a carefully composed compendium of state-of-the-art science in all aspects of research of pancreatic cancer. The experts who were selected to provide contributions are the best in their fields. The content is contemporary and comprehensive. This text is a necessary reference for anyone already doing research in pancreatic cancer.

Again I can only reiterate that I am truly grateful to my colleagues around the world who have worked so hard and so tirelessly to create this reference. Disseminating the scientific breakthroughs that we know now will accelerate the progress that will change the lives of more and more of our patients.

University of California, San Francisco Margaret A. Tempero, M.D.

Preface

Pancreatic cancer has become an even greater challenge today than it was in 2010 while paradoxically seeing exciting progress in its understanding with unforeseen advances in its diagnosis and treatment. The First Edition proved to be a major success with over 30,000 downloads: it was up to date, evincing a deep but manageable exposition of the relevant basic and translational science, complexed within a clinically relevant purpose. The forward momentum meant that a Second Edition became an inevitability, but this has not simply meant just updating the same elemental threads but a reworking of the perspective.

The incidence of pancreas cancer is rising around the world and is predicted to become the second commonest cause of death within a few years. Yet survival is beginning to improve and in the case of potentially curable cases the 5-year survival rates are 30% with postoperative adjuvant combination chemotherapy. By 2012 the estimated global incidence was 337,872 cases per year resulting in 330,391 deaths, and in Europe pancreatic cancer accounted for 103,773 new cases and 104,481 deaths each year. In the USA in 2016, there were around 53,070 new cases of pancreatic cancer diagnosed with 41,780 deaths. The American Cancer Society's estimates for pancreatic cancer in the USA for 2017 were 53,670 (27,970 men and 25,700 women) new cases with 43,090 (22,300 men and 20,790 women) deaths. Pancreatic cancer accounts for about 3% of all cancers in the USA and about 7% of all cancer deaths. The 1-year survival rate of people with pancreatic cancer who do not have surgery has risen from around 10% to 30%, and the overall 5-year survival rate has risen from 5% to 7%. In resected cases, the 5-year survival rates have increased from 8% with surgery alone to 30% with combination adjuvant chemotherapy using gemcitabine and capecitabine.

Advances in surgical techniques now enable many more patients to be operated, and the application of neoadjuvant therapies may also render borderline and locally advanced pancreatic cancers more amenable to resection. But much is still needed to understand the biology of the cancer and how the microenvironment of both the pancreatic primary and its metastases influence this. We have expanded sections on stromal inflammatory cells in pancreatic cancer and tumor-stromal interactions in invasion and metastases. There is enhanced discussion on the management of preneoplastic cystic neoplasms of the pancreas, including the controversial area of intraductal papillary mucinous neoplasms. Advanced technologies for diagnosis and

treatment now encompass cancer exosomes, liquid biopsies and circulating tumor cells, and vaccine therapy and immunotherapy.

The application of pancreatic cancer genetics has been progressed into precision medicine based on next-generation sequencing and master controllers. Other novel therapeutic areas are now expounded in considerable depth including defining pancreatic cancer phenotypes via metabolomics, treatments based on the metabolism of pancreatic cancer, epigenetic pharmacology, differential therapy based on tumor heterogeneity, and multiparameter modalities applied in the setting of individualized medicine.

By drawing on many of the world's recognized scientific authorities and practical clinicians, we hope to inspire the current and future generations of active researchers to make clever and bold decisions on how we can successfully continue the fight against pancreatic cancer.

John P. Neoptolemos
Raul Urrutia
James L. Abbruzzese
Markus W. Büchler

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The Editors would like to dedicate their work to all the pancreatic cancer patients and their families.

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About the Editors

Professor **John P. Neoptolemos** was trained at the University of Cambridge with a double degree in Natural Sciences and Philosophy followed by clinical training at Guy's Hospital in London. He is Professor of Surgery at the University of Heidelberg moving from Liverpool University where he held the Chair of Surgery from 1996 until 2017. As Chairman of ESPAC he successfully led the ESPAC-1, 3, and 4 trials, the results of which have been adopted as guidelines for the treatment of potentially curable pancreatic cancer around the world. As Director, he received the Freedom of the City of Liverpool in 2011 for the Liverpool Cancer Research UK Centre. In 2007, he established the NIHR Liverpool Pancreas Biomedical Research Unit (Scientific Director), the NIHR and Cancer Research UK, Liverpool Experimental Cancer Medicine Centre (Deputy Director), and the Liverpool Clinical Trials and Cancer Research UK Cancer Trials Units (Director). In 2012, he was made an Honorary Member of the German Society of Surgery. In 2013, the Pancreas Unit won the Acute Services Sector Health Services Journal National Award. In 2013, he received a Lifetime Achievement Award from the European Pancreatic Club at its 45th Meeting in Zurich. In 2014, he was made an Honorary Member of the Hungary Society of Surgery and also an Honorary Professor of Nanjing Medical University. He was awarded Hirschberg Award for Pancreatic Cancer, American Pancreatic Association in 2005, and the Ruth Brufsky Award for Pancreas Cancer Research in 2017. Presently, he is on the Medical Advisory Board for Pancreas Cancer UK. He was elected Fellow of the Academy of Medical Sciences in 2007 and NIHR Senior Investigator in 2011.

Dr. Raul Urrutia, M.D., was appointed as Director of the Genomic Sciences and Precision Medicine Center (GSPMC), the Warren P. Knowles Endowed Chair of Genomic Sciences and Precision Medicine, and Professor of Surgery at the Medical College of Wisconsin (MCW) from July 1, 2017. He remains Emeritus Professor at the Mayo Clinic, Rochester, MN, where he previously served as Professor in the Departments of Biochemistry and Molecular Biology, Biophysics and Medicine at the Mayo Clinic College of Medicine in Rochester, MN, and Director of Epigenomics Education and Academic Relationships in the Epigenomics Program, Mayo Clinic Center for Individualized Medicine. Dr. Urrutia was also consultant for the Division of Gastroenterology and Hepatology, Department of Internal Medicine, and consultant (joint appointment) for the Department of Physiology and Biomedical Engineering.

Dr. Urrutia received his M.D. degree from the University of Cordoba Medical School in Cordoba, Argentina, in 1987. During medical school he undertook research in the Cell Biology Institute there, where he published studies on diet-induced genomic changes during the development of pancreatic cancer. From 1987 to 1992, Dr. Urrutia held numerous positions at the National Institutes of Health (NIH), including guest researcher, visiting fellow, visiting associate, and tenure-track visiting scientist, during which he trained in the fields of cell biology, molecular biophysics, protein chemistry, and recombinant DNA techniques.

Dr. Urrutia's career and activities combine interests and expertise in the areas of genomics, epigenomics, and individualized medicine. Since joining the faculty at the Mayo Clinic College of Medicine in 1992, Dr. Urrutia has served as Director of the GI Unit, Director of the Ph.D. Program in Tumor Biology, Associate Director for Genomics at the Mayo Clinic General Clinical Research Center, and Director of the GI Cancer Research Program at the Mayo Cancer Center. Also at Mayo, Dr. Urrutia established the first laboratory dedicated to epigenetics and chromatin dynamics in normal cell populations and in diseases, such as diabetes and pancreatic cancer. His laboratory has discovered diabetes-causing genes such as KLF11, for which its alterations are responsible for juvenile (MODY VII) and neonatal diseases, as well as KLF14 for insulin

resistance metabolic syndrome. Dr. Urrutia's laboratory has made fundamental contributions by identifying members and helping to put together the family of KLF proteins, which are critical regulators of biology, pathobiology, and epigenetic reprogramming. In the area of chromatin and epigenetics, the Urrutia laboratory has discovered new histone deacetylase (HDAC), histone acetyltransferase (HAT), and histone methyltransferase (HMT) epigenetic pathways. His work also led to the discovery of histone-proteins associated subcodes, which helps to interpret epigenomic codes. All of these complexes are associated with the development of cancer, as well as many other diseases, and are currently being explored as an extremely promising area in experimental therapeutics. His work has been continuously funded by the NIH since the early 1990s.

Dr. Urrutia has been past Chair for the Pancreatic Diseases Section for the American Gastroenterological Association (2005–2006), past President for the American Pancreatic Association (2007), and a former member of the board of the International Association of Pancreatology.

James L. Abbruzzese, M.D., is the Chief of the Duke Division of Medical Oncology and serves as the Associate Director for Clinical Research and Training for the Duke Cancer Institute (DCI). Dr. Abbruzzese is a leading expert in the clinical study and treatment of pancreatic cancer, and his management experience and vision for clinical research and the division will substantially support cancer care and research at Duke. Before moving to Duke, he held the Waun Ki Hong Distinguished Chair in Translational Oncology, and he was Chairman of the Department of Gastrointestinal Medical Oncology and Digestive Diseases at the University of Texas M.D. Anderson Cancer Center in Houston.

He earned his medical degree with honors from the University of Chicago, Pritzker School of Medicine, and completed his residency in Internal Medicine at Johns Hopkins Hospital. He also completed clinical fellowships in Infectious Diseases at the Johns Hopkins and in Medical Oncology and Medical Oncology Research Laboratory of Neoplastic Disease Mechanisms at the Dana-Farber Cancer Institute of Harvard Medical

School. Before his recruitment to Duke University he spent most of his professional career at M.D. Anderson, where he progressed through the ranks to assume leadership positions as Chairman of the Department of Gastrointestinal Medical Oncology and Associate Vice-Provost for Clinical Research.

Among his many accomplishments, Dr. Abbruzzese is a Fellow of the American College of Physicians and Fellow of the American Society of Clinical Oncology. He has coauthored more than 400 research publications and is the immediate past Chair of the Clinical Trials and Translational Research Advisory Committee of the National Cancer Institute. He currently serves as the Chair of the NCI Pancreatic Ductal Adenocarcinoma Progress Working Group.

Professor **Markus W. Büchler** is the Executive Director and Professor of Surgery at the Department of Surgery at Heidelberg University. After studying medicine in Heidelberg and Berlin, he started his surgical training at the University of Ulm where he became Deputy Clinical Director in 1987. In 1993, he became Professor of Surgery and Clinical Director of General Surgery at the University of Bern, Switzerland. In 2001, he returned to Germany to start leading the surgical department in Heidelberg. Today, he additionally heads the surgical departments at the Salem Hospital in Heidelberg and at the general hospitals of Sinsheim, Eberbach, and Heppenheim. He is an internationally respected expert in the field of oncologic surgery, especially in the field of pancreatic surgery. Professor Büchler has published more than 2000 scientific papers mainly focused on the translational features of GI cancer and pancreatic diseases as well as clinical surgical problems. Among others he has been President of the German Surgical Society, the European Pancreas Club, and the International Hepato-Pancreato-Biliary Association. He is a member of the German Academy of Sciences Leopoldina and he is an Honorary Member of the American Surgical Association, the American College of Surgeons, and the Royal Colleges of Surgeons of England and Scotland among many other societies. Professor Büchler has received multiple scientific awards, and he is a member of the editorial boards of many well-known scientific journals.

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Part I

The Nature of Pancreatic Cancer

Epidemiology and Prospects for Prevention of Pancreatic Cancer

Patrick Maisonneuve and Albert Lowenfels

Contents

Abstract

Pancreatic cancer, although infrequent, has a very poor prognosis, making it currently one of the fourth or fifth most common causes of cancer mortality in developed countries. Its incidence varies greatly across regions, which suggests that lifestyle factors play an important role in its etiology. Because pancreatic cancer is strongly age dependent, increasing population longevity and aging will lead to an increase of the global burden of pancreatic cancer in the coming decades. In fact, pancreatic cancer is anticipated to move from the fourth to the second leading cause of cancer death in the United States by 2020, despite stable age-specific and age-standardized incidence rates. The increase in pancreatic

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cancer incidence and mortality rates reported in some countries could be largely ascribed to improvement in the diagnosis and ascertainment of the disease, particularly in elderly subjects. The etiology of pancreatic cancer has been extensively studied and has been the subject of numerous meta-analyses and pooled analyses. Using a comprehensive strategy, one can retrieve more than 170 meta-analytical or pooled reports dealing with the association between more than 50 specific risk factors and pancreatic cancer risk. About two-thirds of the major risk factors associated with pancreatic cancer are potentially modifiable, affording a unique opportunity for preventing one of our deadliest cancers: Abstaining from smoking, limiting alcohol intake, adopting a healthy diet, rich in fruits and vegetables, limiting red meat consumption and being physically active in everyday life could reduce pancreas cancer risk by 30 percent.

Keywords
Pancreatic cancer · Epidemiology · Time trends · Etiology · Risk factors · Diagnostic practice · Aging · Preventable fraction · Prevention

Burden of Pancreatic Cancer

Before providing information on pancreatic cancer burden, time trends, or risk factors, it is important to recognize that the term "pancreatic cancer" encompasses distinct types of cancer that are often amalgamated in epidemiological studies [1][i]. Pancreatic cancer can arise either from exocrine or endocrine cells. Exocrine cells produce enzymes such as lipase and amylase and bicarbonate that are secreted into the small intestine to help in digesting foods, while endocrine cells, or islets of Langerhans, produce hormones such as insulin and glucagon to maintain the proper level of sugar in the blood. Tumors of the exocrine pancreas represent more than 95% of all pancreatic cancers and comprise themselves different histological subtypes, adenocarcinomas representing by far the largest group. Endocrine tumors could be benign or malignant and functional (producing hormones) or nonfunctional (producing no hormones) and generally have a better prognosis than exocrine tumors [2]. Because all pancreatic cancer subtypes are regrouped under the same main topographic code of the International Classification of the Diseases (i.e., ICD-9157), it is uncommon to obtain epidemiological data for specific subtypes. Descriptive statistics for pancreatic cancer are however largely driven by adenocarcinomas of the exocrine pancreas, which represent the vast majority of the tumors.

Worldwide, pancreatic cancer could be considered as a rare form of cancer, ranking as the twelfth most common form of cancer, with about 330,000 new cases estimated for both sexes combined in 2012 [3]. Because of its very poor prognosis, approximately the same number of deaths was expected in 2012, placing pancreatic cancer as the seventh most common form of cancer-related death worldwide, both in men and in women. Pancreatic cancer incidence and mortality vary significantly across major world areas. In developing countries, pancreatic cancer

represents a rare disease, ranking respectively as the eighth and tenth most common cause of cancer-related death in men and in women. This lower incidence could be largely attributed to the short life expectancy observed in these countries and to poor diagnostic assessment. In fact, we will see in the following sections that pancreatic cancer is very strongly related to age and that its accurate diagnosis relies on modern technologies not often available in low-income countries. In contrast, in developed countries, pancreatic cancer is an appreciable form of cancer, representing about 3% of all cancer cases and 6% of all cancer-related deaths. In some westernized countries such as the United States of America [4], pancreatic cancer ranks as the fourth most common cause of cancer-related death both in men after lung, prostate, and colorectal cancer and in women after lung, breast, and colorectal cancer.

Overall more than a fivefold difference in age-standardized rates is observed between low- and high-incidence countries (Fig. 1). Age-standardized (world standard population) incidence rates in less-developed countries were estimated as 3.3 per 100,000 in men and 2.4 per 100,000 in women for 2012 [3]. For the same year, the country with the highest estimated incidence of pancreatic cancer was the Czech Republic with an age-adjusted rate of 11.9 per 100,000 in men and 7.9 per 100,000 in women. Most of the other countries with high incidence rates are located in Central or Eastern Europe for both sexes and in Scandinavia for women (Table 1). High rates are also recorded in Japan in contrast with those of other countries from Asia.

Time Trends and Related Factors

Variations in cancer incidence may result from several factors well described in a paper by Weir et al. [5]. These factors could be regrouped in three different large categories: (1) change in cancer risk or diagnostic practice, (2) population growth, and (3) population aging. While population growth is more directly related to the variation in the absolute number of cancer diagnosed in a country and is specific to that country, the two other factors are particularly pertinent to pancreatic cancer. Change in cancer risk may a priori be considered as the most obvious factor: A good example is the dramatic change in lung cancer incidence that parallels the epidemic of tobacco consumption [6]. But, unlike lung cancer which is largely caused by exposure to tobacco smoke, pancreatic cancer is a multifactorial disease. Many of its risk factors have been identified (see following section), but altogether they explain only a fraction of all the cases [7]. Since exposure to these risk factors often varies in different ways (i.e., decreasing smoking consumption offset by an increasing prevalence of obesity or diabetes), it is unlikely that changes in the prevalence of a single risk factor could alone explain variations in pancreatic cancer incidence within or across countries.

Change in Diagnostic Practice

On the contrary, change in diagnostic practice is or has been in the past, an important factor. Pancreatic cancer is in fact very difficult to diagnose. Most patients remain

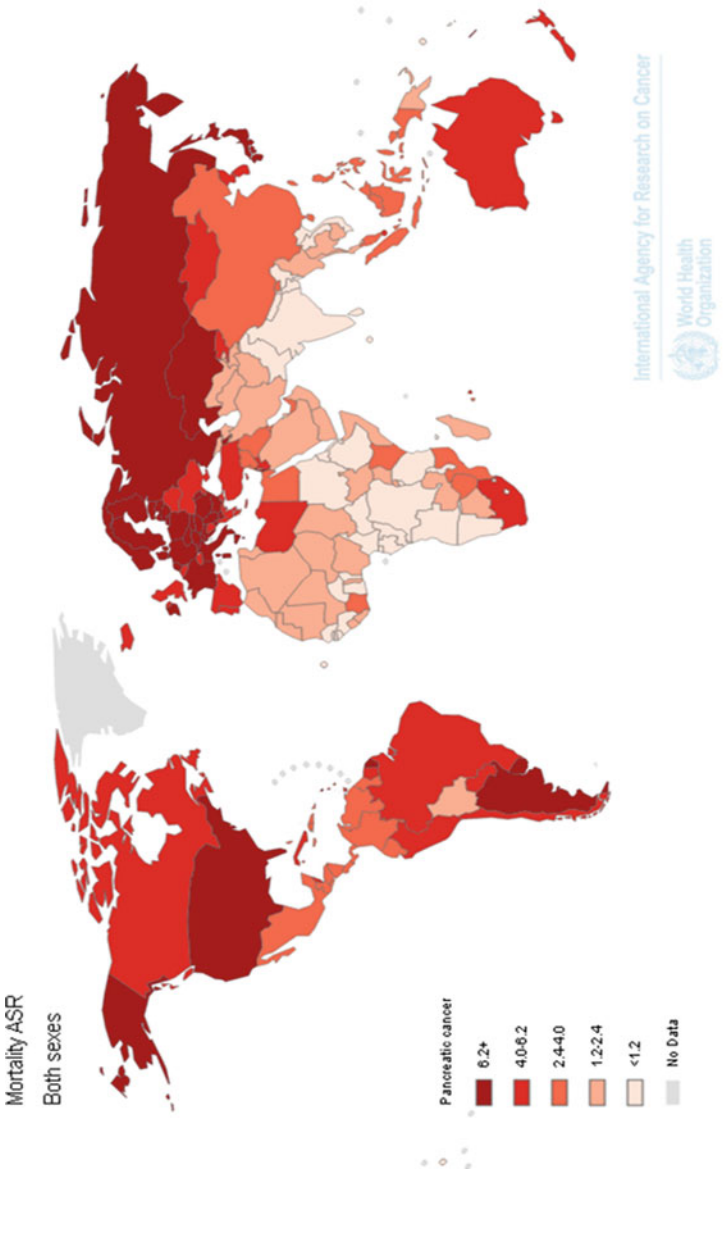

Fig. 1 Estimated age-standardized mortality rates (*ASR*) for pancreatic cancer in 2012 for both sexes combined

Table 1 Pancreas cancer incidence rates (GLOBOCAN estimates for 2012)

Males	Cases	ASRW	Females	Cases	ASRW
World	178,161	4.9	World	159,711	3.6
Less developed	83,459	3.3	Less developed	66,948	2.4
More developed	94,702	8.6	More developed	92,763	5.9
Countries with the highest incidence rates					
Czech Republic	1,086	11.9	Czech Republic	1,032	7.9
Armenia	214	11.9	Slovenia	207	7.8
Slovakia	440	11.5	Slovakia	441	7.8
Hungary	906	11.5	Denmark	510	7.7
FYR Macedonia	173	11.5	Finland	596	7.6
Latvia	174	10.8	Hungary	950	7.4
Japan	17,013	10.6	Armenia	217	7.3
Lithuania	246	10.6	Germany	8,479	6.9
Bulgaria	686	10.4	Austria	800	6.9
Romania	1,692	10.3	Japan	15,886	6.7

free of symptoms until the disease has reached an advance stage. Its location is difficult to access, and the advanced age of the patients at diagnosis also contributes to the limited proportion of those amenable to resection. Palliative treatment remains the only option in many cases and frequently no histological confirmation of the cancer is available. In the absence of surgical specimen, an accurate diagnosis of pancreatic cancer relies on fairly modern and expensive imaging instrumentations and demanding procedures including endoscopic retrograde cholangiopancreatography (ERCP) introduced in the late 1960s, computed tomography (CT) in the mid-1970s [ii], endoscopic ultrasound (EUS) in early 1980s, magnetic resonance cholangiopancreatography (MRCP) [iii], or endoscopic ultrasound fine needle aspiration (EUS-FNA) in the early 1990s [8][iv]. Without these diagnostic tools, many pancreatic cancer cases in the past or even at present in low-income countries, particularly those presenting as diffuse metastatic disease or in elderly or debilitated people, might have been improperly diagnosed or reported. Registration of cancer incidence and mortality also depends upon coding instruments such as the International Classification of Disease (ICD) of the World Health Organization (WHO) [9, 10][v]. This classification has itself evolved overtime, and several alternative specifications to "Malignant neoplasm of pancreas" could have been used to account for imprecision or uncertainty of the diagnosis including "Malignant neoplasm of other and ill-defined sites within the digestive organs," "Malignant neoplasm of other and ill-defined sites," "Secondary malignant neoplasm of digestive systems," "Malignant neoplasm without specification of site," or even "Neoplasm of unspecified nature" (Table 2). Part of the increase in cancer incidence and mortality observed until the 1990s in westernized countries, still ongoing in developing countries or in the older age groups could be ascribed to change in diagnostic practice. This is further supported by data showing increase of the percentage of pancreas cancers that were staged and diagnosed histologically in the last decades in parallel with data

Table 2 International classification of diseases

ICD-6 (1948-)	ICD-7 (1955-)	ICD-8 (1965-)	ICD-9 (1975-)	ICD-10 (1990-)
157 Malignant neoplasm of pancreas	**157** Malignant neoplasm of pancreas	**157** Malignant neoplasm of pancreas	**157** Malignant neoplasm of pancreas	**C25** Malignant neoplasm of pancreas
159 Malignant neoplasm of unspecified digestive organs	**159** Malignant neoplasm of unspecified digestive organs	**159** Malignant neoplasm of unspecified digestive organs	**159** Malignant neoplasm of other and ill-defined sites within the digestive organs and peritoneum	**C26** Malignant neoplasm of other and ill-defined digestive organs
			195 Malignant neoplasm of other and ill-defined sites	**C76** Malignant neoplasm of other and ill-defined sites
198 Secondary and unspecified malignant neoplasm of lymph nodes	**198** Secondary and unspecified malignant neoplasm of lymph nodes	**197** Secondary malignant neoplasm of respiratory and digestive systems	**197** Secondary malignant neoplasm of respiratory and digestive systems	**C78** Secondary malignant neoplasm of respiratory and digestive organs
199 Malignant neoplasm of other and unspecified sites	**199** Malignant neoplasm of other and unspecified sites	**199** Malignant neoplasm without specification of site	**199** Malignant neoplasm without specification of site	**C80** Malignant neoplasm without specification of site
230 Neoplasm of unspecified nature of digestive organs	**230** Neoplasm of unspecified nature of digestive organs	**230** Neoplasm of unspecified nature of digestive organs		**D37** Neoplasm of uncertain or unknown behavior of oral cavity and digestive organs
239 Neoplasm of unspecified nature of other and unspecified organs	**239** Neoplasm of unspecified nature of other and unspecified organs	**239** Neoplasm of unspecified nature of other and unspecified organs	**239** Neoplasm of unspecified nature	**D48** Neoplasm of uncertain or unknown behavior of other and unspecified sites

showing decrease of the number of cases recorded as "cancer of unknown primary" [11, 12]. Analysis of time trends after the introduction and diffusion of appropriate diagnostic tools and excluding people in the older age groups which are subject to less intensive diagnostic work-up should provide more accurate information on the real variation of pancreatic cancer incidence over time.

Population Aging

Aging of the population represents another major factor responsible for the increase of the number of cancer cases diagnosed in developed countries [13]. This is

particularly true for pancreatic cancer which is strongly age dependent, being exceptionally diagnosed before age 40 and uncommon before age 50. In an aging country like Italy, nowadays no more than 10% of all pancreatic cancer cases are diagnosed before age 60, approximately 55% between age 60 and 80, and remarkably 35% (still increasing) in elderly people aged 80 years or more. This is a consequence of the astonishing increase in life expectancy that occurred in Italy, or in other westernized countries, over the last century, jumping from 35 years in the late 1800s to over 80 years currently. With the continuing decline of mortality from the big killers (cardiovascular disease and major forms of cancer) [14], the proportion of patients reaching an age at which pancreatic cancer is more frequent will continue to increase.

Time Trends

Analysis of long-time series such as data available from 1930 for the United States shows a modest increase of age-adjusted pancreatic cancer death rates over time in both sexes, contrasting with the clear reduction of mortality from the major forms of cancer such as stomach, uterine cervix, colorectal, prostate, breast, and more recently lung cancer. This rising trend is common to many countries worldwide, but the extent of the increase varies from country to country: Higher increases are seen for countries with historically low mortality rates of pancreatic cancer such as Spain, Italy, Greece, or Japan, while the increase is less important for countries with historically high mortality rates such as the United States, the United Kingdom, Sweden, or Norway (Fig. 2). This points to an ostensible globalization of pancreatic cancer mortality rates, more evident in men than in women, which could in part result from the standardization of diagnostic and coding practice. Since the analysis is based on age-standardized rates, aging of the population is unlikely to explain any of the variation observed.

The pattern is somewhat different when focusing on age-adjusted incidence or mortality rates recorded in the last two decades in countries with large access to modern diagnostic technology. During this more recent period, no significant trends are generally observed in men or in women (National cancer registry of Ireland, Cancer Research UK, US SEER). An analysis of time trends based on age-specific rates instead of age-adjusted rates reveals that pancreatic cancer rates are stable over time in all 5-year age groups below age 70 or 75, but an increase is observed in older age groups particularly in those aged 80 or more. The residual increase in older age groups could again be attributed to change in diagnostic practice among elderly as well as to the reduction of competing cause of death from other forms of cancer and cardiovascular disease. This pattern is present in many countries including the United States or Italy but is particularly evident for Japan, with a drastic increase of age-specific pancreatic cancer mortality rates for all age groups from 1955 until the mid-1980s that stabilized afterward except for elderly subjects for whom a slight increase is still apparent (Fig. 3).

Despite stable age-adjusted rates, the number of pancreatic cancer cases and deaths is drastically increasing in most westernized countries. In 2014, Rahib et al. [15]

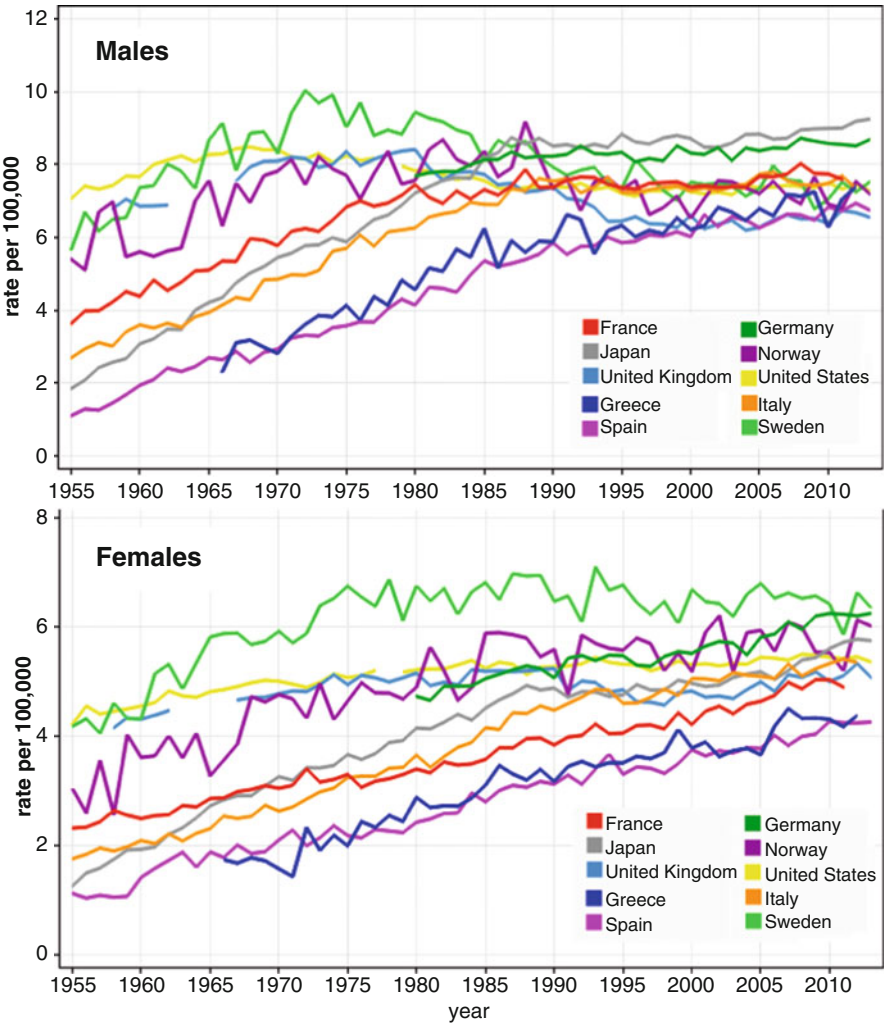

Fig. 2 Trends in age-adjusted (world standard population) pancreatic cancer death rates in selected countries worldwide (Source: International Agency for Research on Cancer (Lyon, France))

published projections of cancer incidence and deaths to 2030 in the United States. They found an "unexpected" burden of pancreas cancers which are anticipated to move from the fourth to the second leading cause of cancer-related death in the United States by 2020. Likewise, Ferlay et al. [16] estimated that by the year 2017, more deaths from pancreatic cancer will occur than breast cancer in the European Union and that pancreatic cancer may become the third leading cause of death from cancer in the EU after lung and colorectal cancers. This increase of the absolute number of cases and deaths is a direct consequence of aging of the population. From a simple projection, applying pancreatic cancer age-specific rates reported for Italy in 2010 to the projected

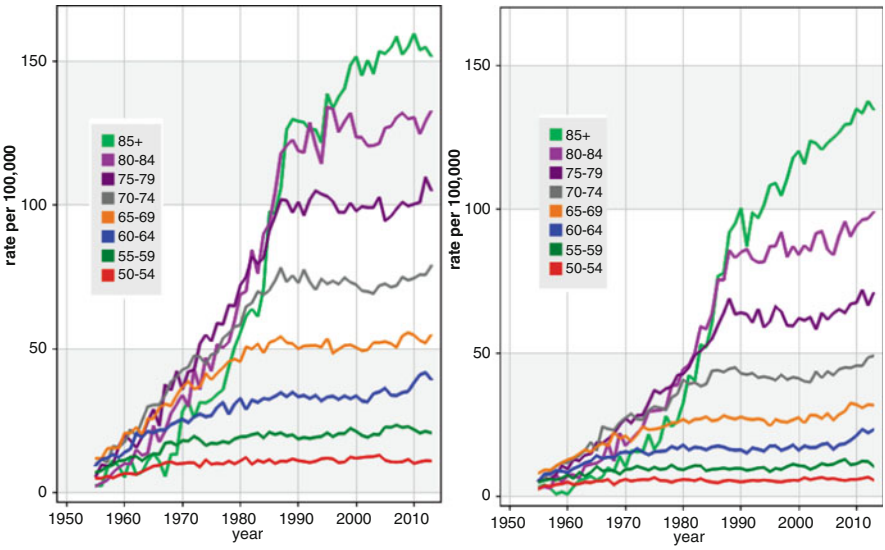

Fig. 3 Trends in age-specific pancreatic cancer death rates in Japan (Source: International Agency for Research on Cancer (Lyon, France))

Table 3 Projection of pancreatic cancer deaths in Italy

Males	**2000**	**2010**	**2020**	**2030**	**2040**	**2050**	**Trend**
50–59	536	498	615	582	450	425	=
60–69	1,109	1,206	1,347	1,651	1,566	1,228	=
70+	2,122	3,195	4,005	4,809	5,987	6,790	↗
Females	**2000**	**2010**	**2020**	**2030**	**2040**	**2050**	**Trend**
50–59	299	333	396	364	278	259	=
60–69	841	873	960	1,145	1,056	811	=
70+	3,197	4,012	4,835	5,618	6,755	7,672	↗

Italian population obtained from the Population Division of the United Nations, the number of pancreatic cancer cases in Italian subjects aged 70 or more is predicted to increase from ~7,200 in 2010 to ~8,800 (+22%) in 2020 and >10,400 (+44%) in 2030, while the number of cases aged less than 70 will be about constant and this despite fixed age-specific incidence rates (Table 3).

Risk Factors

The list of suspected risk factors for pancreatic cancer is very long and has been subject of a dedicated chapter in the previous edition of this book [17]. Unlike lung or uterine cervix cancers which are mostly caused by exposure to a single risk factor (respectively, tobacco smoke and human papilloma virus infection), pancreatic

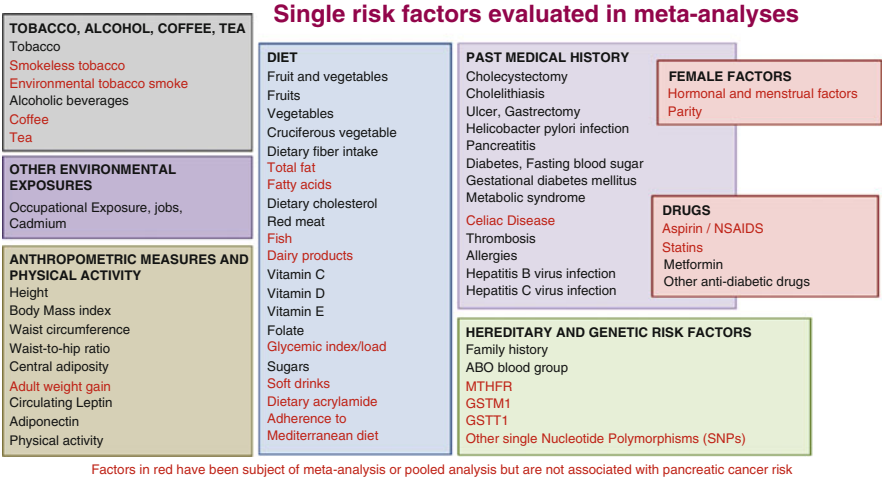

Fig. 4 Single risk factors evaluated in meta-analyses. Factors in *red* have been subject of meta-analysis or pooled analysis but are not associated with pancreatic cancer risk

cancer has been associated with numerous risk factors involved in several distinct pathways [7]. The recent proliferation of systematic reviews, meta-analyses, and pooled analyses of results or individual data from published reports had allowed to identify, quantify, and summarize the association between a series of uncommon or modest risk factors that could not have been possible in a single observational study. A preliminary summary review based on results from 117 meta-analyses or pooled analyses was a comprehensive strategy; one can 1 year later retrieve more than 170 meta-analytical or pooled reports dealing with the association between more than 50 specific risk factors and pancreatic cancer risk (Fig. 4). For many of the risk factors, a statistically significant association has been established, while for a series of other studied risk factors, the results from meta-analyses ascertain the lack of an association with pancreatic cancer.

Hereditary and genetic factors are responsible for a small proportion of pancreatic cancer cases [18]. A family history of pancreatic cancers approximately double the risk of pancreatic cancer, and it is estimated that 5–10% of patients with pancreatic cancer have an underlying germline disorder [19]. Having a non-O blood group, another inherited characteristic, has also been steadily associated with an increased risk of pancreatic cancer [20][vi]. Tobacco smoking is the most important and established lifestyle-related risk factor, being responsible for approximately 20% of all pancreatic tumors [21]. Although a common cause of pancreatitis, heavy alcohol intake is associated only with a modest increased risk of pancreatic cancer [22]. Many factors associated with the metabolic syndrome, including overweight and obesity [23], impaired glucose tolerance [24], and long-standing diabetes [25] also increase the risk disease, while atopic allergy [26] and use of metformin [27] as a treatment for diabetes have been associated with a reduced risk of pancreatic

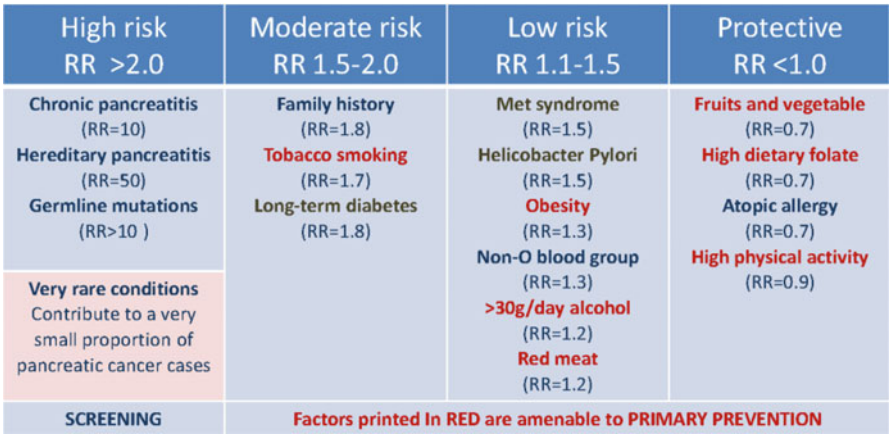

Fig. 5 Major risk factors for pancreatic cancer

cancer. Other medical conditions such as a history of chronic pancreatitis [28] or cholecystectomy [29], infection with *Helicobacter pylori* [30], hepatitis B [31], or hepatitis C [32] virus also increase the risk of developing pancreas cancer. There is only limited evidence of an association between diet and pancreatic cancer risk, with an apparent modest increased risk with increasing red meat [33] and processed meat [34] consumption and risk reduction with increasing consumption of fruits and vegetables [35] and folate [36]. Several meta-analyses confirmed no association with either coffee or tea consumption [37], with total fat [38], dairy products [39], dietary acrylamide [40], or fish intake [41], with glycemic index or glycemic load [42], with exposure to smokeless tobacco [43] or environmental tobacco smoke [44].

Only very few of these risk factors are associated with relative risks greater than two and could be used for identifying individuals who could benefit from screening. These include individuals with a strong family history of pancreatic cancer, with a history of chronic or hereditary pancreatitis, or who have another genetic predisposition for developing this disease (Fig. 5). Although tobacco smoking and long-standing diabetes are among the most established risk factor, the magnitude of the association is only moderate, being comprised between 1.5 and 2.0. Most of the remaining risk factors could be considered "low risk" with summary relative risk never exceeding 1.5.

Prospects for Prevention

The increasing number of pancreatic cancer cases, the difficulty to diagnose the tumor at an early stage, and the lack of effective treatment make primary prevention one of the best options to reduce burden of the disease. Many of the identified risk factors for pancreatic cancer are definitely amenable to primary prevention, but the

magnitude of their association is only low or moderate (Fig. 5). Tobacco smoking alone is probably responsible for 20–30% of all pancreatic cancer cases [7, 45–46]. Because heavy alcohol consumption is associated with a modest 20% increased risk of pancreatic cancer and because the prevalence of heavy (>30 g/day) drinkers is low in the general population, alcohol is thought to be responsible for less than 10% of all pancreatic cancer cases [47]. Depending of the country, the attributable fraction of obesity varies from 3% to as optimistic as 27% for the US population [48]. Again the attributable fraction of high meat or low fruit intake is small [7]. Parkin et al. [49] estimated that 36% of pancreatic cancers were attributable to lifestyle and environmental factors in the United Kingdom in 2010.

It is remarkable that most of the preventable risk factors for pancreatic cancer are also associated with other forms of cancer or with other non-neoplastic diseases. This is the case for tobacco smoking, excessive body weight, lack of physical activity, unhealthy diet, or excessive alcohol consumption. Therefore, the general recommendations already in place for cancer prevention apply particularly well to pancreatic cancer. For instance, the top 6 of the 12 recommendations of the European Code against Cancer have a direct impact on pancreatic cancer [50]. These recommendations are:

1. *Do not smoke. Do not use any form of tobacco.*
2. *Make your home smoke-free. Support smoke-free policies in your workplace.*
3. *Take action to be a healthy body weight.*
4. *Be physically active in everyday life. Limit the time you spend sitting.*
5. *Have a healthy diet:*
   - *Eat plenty of whole grains, pulses, vegetables, and fruits.*
   - *Limit high-calorie foods (foods high in sugar or fat) and avoid sugary drinks.*
   - *Avoid processed meat; limit red meat and foods high in salt.*
6. *If you drink alcohol of any type, limit your intake. Not drinking alcohol is better for cancer prevention.*

It is difficult to quantify how many pancreatic cancer cases the adoption of these measures could prevent, but a study based on data from the prospective National Institutes of Health-AARP Diet and Health Study provides exciting information [51]. This study followed about 450,000 participants aged 50–71 years and 1,057 incidental pancreatic cancer cases were observed during follow-up. Participants were retrospectively scored on five modifiable lifestyle factors as healthy or unhealthy, receiving 1 point for each respective lifestyle factor: nonsmoking, limited alcohol use, adherence to the Mediterranean dietary pattern, body mass index ($\geq$18 and <25), or regular physical activity. The authors estimated that as much as 30% of pancreatic cancer cases in this study were attributable to low healthy lifestyle scores (0–3) and could have been prevented. Because action for tobacco control, for the limitation of alcohol consumption, and for weight control and action plan for healthy diet and exercise are difficult to perform and reach only limited success, a much lower proportion of pancreatic cancer could however be prevented in the real life.

Conclusion

Pancreatic cancer represents a major cause of cancer-related deaths. Part of the increase seen in the past could be ascribed to change in diagnostic and coding practices, while aging of the population is at large responsible for the increase of the number of cases and deaths observed today particularly among elderly individuals. Pancreatic cancer is a multifactorial disease: Hereditary and genetic factors are associated with high risks of developing cancer but are responsible for a small fraction of the cases. Many of the environmental and lifestyle risk factors for pancreatic cancer are common risk factors to other forms of cancer and non-neoplastic diseases and are preventable. Adoption of a healthy lifestyle could substantially reduce pancreatic cancer burden.

Cross-References

- ▶ MRI and MRCP for Diagnosis and Staging of Pancreatic Cancer
- ▶ New Japanese Classification of Pancreatic Cancer
- ▶ Pancreatic Adenocarcinoma: CT and PET/CT
- ▶ Pathologic Classification and Biological Behavior of Pancreatic Neoplasia
- ▶ Secondary Screening for Inherited Pancreatic Ductal Adenocarcinoma

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Cell Cycle Machinery and Its Alterations in Pancreatic Cancer

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Abstract

Cancer is a disease of uncontrolled cell proliferation. Sequencing of the pancreatic cancer genome revealed frequent gene alterations that lead to constitutive proliferation signals and loss of the breaking systems. Cancer cells also display defects in the DNA repair systems, which suggest that compromised genome integrity contributes to the tumorigenesis process. These observations explain many of the abnormal behaviors of cancer cells, yet stopping proliferation of cancer cells remains a difficult task.

This chapter will describe misregulation of the cell-cycle machinery in pancreatic cancer and therapeutic options to stop abnormal proliferation. The basic concept of the normal cell cycle will be outlined first, and the mechanisms of DNA repair will be introduced. Next, alterations of the cell cycle and DNA repair systems in pancreatic cancer will be described. Finally, therapeutic opportunities to target the specific alterations in the cell cycle and DNA repair systems in pancreatic cancer will be discussed.

Keywords

Cell cycle · CDK · Cyclin · pRB · DNA damage · Checkpoint · p53 · DNA repair · BRCA

Phases of the Cell Cycle

The cell cycle is a process of cell duplication and division, in which two daughter cells are produced from one mother cell. The very essence of the cell cycle is duplication and segregation of the genetic information, which is stored as a form of DNA sequence in the genome. Complementary pairing of the bases in DNA duplexes underlies the copying mechanism of the genome (i.e., DNA replication), in which two identical duplexes are reproduced using each strand of unwound DNA duplex as a template. Duplication of the genome is followed by mitosis, where two copies of the genetic information are segregated into separate daughter cells. Thus, the cell cycle is alternating cycles of the DNA synthesis phase (S phase) and the mitotic phase (M phase) (Fig. 1). The gap between M and S phase is called G1 phase, whereas the gap between S and M phase is called G2

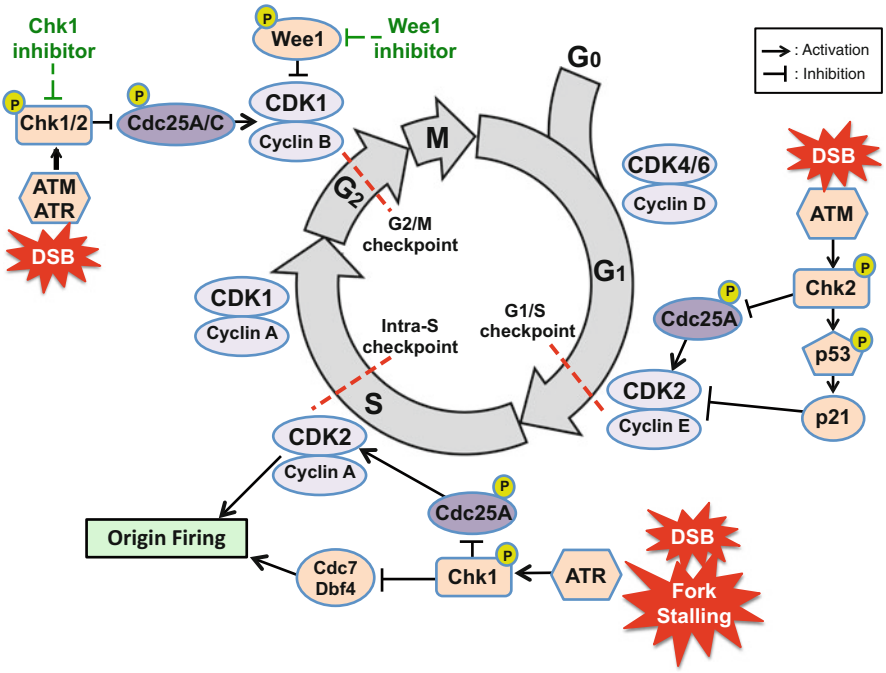

Fig. 1 The cell cycle and DNA damage checkpoints. The cell cycle, which comprises four phases, G1, S, G2, and M, is primarily driven by cyclin-dependent kinases (CDKs) and its binding partners, cyclins. Cdc25 phosphatases remove inhibitory phosphorylation from CDKs and promote cell-cycle progression. In response to DNA damage, cells rely on three major cell-cycle checkpoints: G1/S, intra-S, and G2/M. In response to double strand breaks (DSBs) in G1 phase, activated ATM phosphorylates and activates the downstream kinase Chk2, which in turn phosphorylates Cdc25A and inhibits Cdc25A-mediated activation of CDK2-cyclin E, leading to G1 arrest. Activated ATM and Chk2 also promote stabilization of p53, which in turn induces transcriptional induction of $p21^{Cip1}$, which inhibits CDK2 and prevents S-phase entry. The intra-S checkpoint is primarily mediated by the ATR kinase and is activated by replication fork stalling and resected DSBs, both of which generate single-strand DNA. Activated ATR phosphorylates and activates Chk1, which in turn phosphorylates Cdc25A and inhibits Cdc25A-mediated activation of CDK2. Chk1 also inhibits the Cdc7-Dbf4 kinase. The inhibition of Cdc7-Dbf4 and CDK2, which are required for origin firing, leads to S-phase arrest. When DSBs occur in late S or G2 phase, activated ATM/ATR activate Chk2/Chk1, which in turn phosphorylate and inhibit Cdc25A/Cdc25C-mediated activation of CDK1-cyclin B, leading to G2 arrest. Chk1-mediated phosphorylation stabilizes Wee1, which also contributes to G2/M arrest by maintaining the inhibitory phosphorylation on CDK1. Potential therapeutic drugs (Chk1 and Wee1 inhibitors) are also indicated. *P* phosphorylation

phase. After mitosis, cells make a decision in G1 phase as to whether they continue to proliferate, or exit the cell cycle and enter a quiescence state termed G0 phase. The decision depends on whether the cell receives mitogenic or differentiation signals in G1 phase.

G1 Phase

DNA replication requires sequential assembly of the replication machinery on chromosomes [1]. This process starts in G1 phase with the loading of two hexametric rings of MCM2-7, a replicative helicase, at origins of replication. This is achieved in a concerted action of origin recognition complexes (ORCs), CDC6 and CDT1, which are thought to work as an ATP-dependent clamp loader. The complex containing ORCs, CDC6, CDT1, and MCM2-7 is termed prereplicative complexes (pre-RCs) and marks potential sites of replication initiation. Pre-RC formation in G1 phase also licenses origins for replication initiation in the subsequent S phase. The double hexamer of MCM2-7 complexes remain inactive as a helicase until S phase because they lack cofactors.

S Phase

Upon S-phase entry, the replicative helicases are activated via loading of CDC45 and GINS on MCM2-7 [1]. Resulting is two active CMG (*C*DC45, *M*CM2-7, *G*INS) helicases that encircle each single-strand DNA (ssDNA) and unwind DNA duplexes in the 3′–5′ direction. Once origins are fired and DNA duplex unwound, exposed ssDNA is coated with ssDNA-binding proteins RPAs, and the DNA polymerase α (Pol α)-primase complexes synthesize RNA/DNA hybrid primers. The primer synthesis allows loading of PCNA, a processivity factor for replicative polymerases Pol δ or Pol ε, by the RFC clamp loader composed of RFC1-5. PCNA loading at the primer-template junctions allows polymerase switching from Pol α to replicative polymerase Pol δ or Pol ε. Because DNA duplex is antiparallel, DNA synthesis is continuous on one strand (leading strand), while discontinuous on the other strand (lagging strand). The discontinuous DNA segments in the lagging strand, called Okazaki fragments, are joined together by DNA ligase to form a continuous DNA strand.

While chromosomes are replicated, a pair of replicated chromosomes (called sister chromatids) is held together with cohesin, a ring-shaped protein complex that encircles DNA, until two sister chromatids are separated in mitosis [2]. The loading of cohesin in S phase is performed by the RFC CTF18 lamp loader, which has the same subunits as the PCNA clamp loader RFC except the RFC1 subunit is replaced with CTF18.

G2 Phase

Faithful chromosome segregation in mitosis is dependent on successful completion of DNA replication. In G2 phase, cells ensure that DNA replication is finished throughout the genome, and block the onset of mitosis if there are unreplicated DNA segments (see Sect. 4.2.3). Because sister chromatids can become entangled after DNA replication, cells start decatenating sister chromatids in G2 phase by using

topoisomerase II, which cuts DNA duplex and passing another through the break in an ATP-dependent manner.

M Phase

Mitosis can be described as five distinctive phases: prophase, prometaphase, metaphase, anaphase, and telophase. In prophase, chromosome condensation is initiated with condensin II, which promote loop formation of chromosomes [3]. Concomitantly, cohesin rings dissociate from chromatin, except for at the centromere, where cohesins remain on chromatin until anaphase [2]. Two centrosomes begin to move apart and spindle microtubules are formed. In prometaphase, the nuclear envelope is broken down and, condensin I, another type of condensin that is cytoplasmic in the interphase, gains access to the chromosomes. Condensin I compacts chromosomes further into well-defined rod-shaped structures. In addition, spindle microtubules are attached to sister chromatids at the centromeric structure called kinetochore. In metaphase, all sister chromatids are aligned at the midpoint of the two centrosomes. At the onset of anaphase, ubiquitin E3 ligase APC/C^{Cdc20} promotes degradation of securin, an inhibitor of cysteine protease called separase [4]. Upon securin degradation, activated separase cleaves cohesins remaining at the kinetochore, allowing movement of each sister chromatid toward the opposite spindle poles. Finally in telophase, spindles are disassembled and chromosomes are decondensed. At the same time, new nuclear envelopes are formed around segregated chromosomes in two future daughter cells, and cytokinesis is completed with the division of the cytoplasm.

Successful segregation of sister chromatids requires the attachment of all kinetochores to microtubules and formation of bipolar spindles. Cells ensure these by a mechanism called spindle checkpoint, which monitors proper attachment of microtubules to the kinetochore [4]. In prometaphase and metaphase, kinetochores without microtubule attachment generate diffusible signals of a protein called Mad2 to prevent activation of APC/C^{Cdc20}. Because one unattached kinetochore is sufficient to generate a signal to block APC/C activation, progression to anaphase is prevented essentially until all kinetochores have attached spindles.

Regulation of CDKs

The cell cycle is driven by a signaling cascade of protein phosphorylation. The major driving force is a family of enzymes called cyclin-dependent kinases (CDKs). CDKs require interaction with regulatory subunits, called cyclins, to become active. Levels of most cyclin proteins fluctuate in the cell cycle, and that is reflected in the oscillation of CDK activity in the cell cycle. In addition to cyclin binding, CDKs undergo regulation by phosphorylation and interaction with CDK inhibitors, which

help establish switch-like activation mechanisms, or stop cell-cycle progression if necessary.

Phosphorylation by CDKs has wide variety of effects on substrates, including activation or inhibition of enzymes and induction of protein-protein interactions. CDKs phosphorylate a large number of substrates that are directly involved in duplication and segregation of chromosomes. CDKs also phosphorylate many regulators of cell-cycle events, thereby acting as master regulators of the cell cycle.

CDKs and Cyclins

CDKs

CDKs are serine/threonine kinases that play a central role in cell-cycle progression. There are four major CDKs involved in the cell-cycle regulation. CDK4 and CDK6 are the G1 CDKs that are necessary to enter the cell cycle from the quiescent state. CDK2 is responsible for S-phase entry and promoting DNA replication, whereas CDK1 is the mitotic CDK that drives mitosis in M phase.

CDKs are proline-directed kinases that phosphorylate serine or threonine residue followed by proline in the context of [S/T]PX[K/R], where S/T indicates the serine or threonine residue that is phosphorylated by CDKs, PX indicates proline followed by any amino acids, and K/R indicates lysine or arginine residues. The levels of CDK proteins are mostly stable in the cell cycle, so the regulation of the activity comes in part from the changes in the abundance of the regulatory partner, the cyclin proteins.

The active site of CDKs is located in a cleft where ATP is bound deep inside. In monomeric CDKs, the catalytic cleft is blocked by a segment called the T loop. To achieve full activation, CDKs require cyclin-binding and phosphorylation of the T loop by CDK-activating kinases (CAKs).

Cyclins

Distinct types of cyclins are expressed in different phases of the cell cycle, and they can be classified into four types based on the phases they function in (Fig. 1). Cyclin D is the G1 cyclin that partners with CDK4 or CDK6 to promote cell-cycle entry. Cyclin D gene transcription reflects the mitogenic stimuli and transmits the growth signal to the cell-cycle machinery by inducing cyclin E and cyclin A transcription. Cyclin E is the G1/S cyclin that triggers S-phase entry together with CDK2, while cyclin A is the S cyclin that forms a complex with either CDK2 in S phase or CDK1 in G2/M phase. DNA replication in S phase is driven by CDK2-cyclin E and CDK2-cyclin A. Finally, cyclin B is the M cyclin that is responsible for driving mitotic events in M phase.

Except for the D-type cyclins, the levels of cyclin proteins fluctuate during the cell cycle due to their degradation at specific phases of the cell cycle. Cyclin E is degraded in S phase after phosphorylation by CDK2-cyclin A, which generates binding site for the E3 ubiquitin ligase SCF^{Fbw7}. In contrast, cyclin A and cyclin B

contains a motif called destruction box, which can be recognized by another ubiquitin E3 ligase APC/C^{Cdc20} [5]. In early mitosis, CDK1-cyclin A phosphorylates APC/C subunits and promotes Cdc20 binding with APC/C, thereby inducing its own degradation. Cyclin B is degraded in anaphase after spindle checkpoint has confirmed that microtubules are attachment to all the kinetochores [4].

Regulation of cyclin proteins by proteolysis has several advantages over regulation at the transcriptional level. One is the ability to rapidly shut off CDK activity. This cannot be achieved easily by transcriptional regulation, because proteins will remain even after transcription is turned off. Another advantage is the irreversible nature of protein degradation. By the rapid destruction of the regulatory subunits, cells achieve tight control of CDK activity and ensure that the cell cycle does not move backwards.

Activation Mechanisms of CDKs

Binding with Cyclins

Cyclins interact with specific CDK partners through its conserved cyclin box. Interaction with cyclins induces a structural change of CDKs and promotes a shift to an active conformation. Upon cyclin-binding, the T loop, which is located at the entrance of the catalytic cleft, is moved away from the substrate-binding site. The ATP-binding site in the cleft also undergoes conformational changes, in which the active site residues are realigned and ATP is correctly oriented.

Cyclins also provide substrate specificity to the CDK partner. For example, in the case of CDK2-cyclin E and CDK2-cyclin A, cyclins directly bind to substrates via the Cy (or RXL) motif, which interacts with the hydrophobic patch of the cyclin proteins. Together with the consensus sequence of phosphorylation site ([S/T]PX[K/R]), the Cy motif constitutes a bipartite substrate recognition motif for CDK2-cyclin E and CDK2-cyclin A.

T-Loop Phosphorylation of CDKs

Full activation of CDKs requires phosphorylation of the T loop by CAK. In the case of CDK2, the phosphorylation site is Thr160. The phosphate group on this residue is bound by three Arg residues coming from different parts of CDK2 and induces conformational changes that result in increased substrate binding.

The ternary complex containing CDK7, cyclin H and Mat1 is the CAK for all the cell-cycle CDKs [6]. CDK4 and CDK6 appear to require cyclin binding prior to CAK-dependent T loop phosphorylation, while CAK acts on monomeric CDK2 before cyclin binding. In the case of CDK1, cyclin binding and T loop phosphorylation are interdependent.

The phosphorylated T loop of CDK2 and CDK1 is protected from phosphatases, so that CAK is only necessary to establish active CDK2 and CDK1. On the contrary, the phosphate group on the T loop of CDK4 and CDK6 is unprotected, so that continuous CAK activity is necessary to maintain active CDK4 and CDK6. This indicates that CDK4 and CDK6 are more likely to reflect the changes in CAK

activity. At the G0-G1 transition, CDK7 activation coincides with CDK4 T loop phosphorylation and CDK4 activation. Thus, CAK-mediated T loop phosphorylation appears to play a regulatory role in CDK4 and CDK6 activation, transmitting changes in mitogenic signals to the cell-cycle machinery.

Inhibitory Phosphorylation of CDKs

CDK1 and CDK2 can be inhibited by phosphorylation at Tyr15 by the Wee1 kinase. The mechanism of CDK inhibition by the modification is through the blockade of substrate peptide binding and the induction of nonproductive conformation of the γ-phosphate group of ATP in the catalytic cleft. The phosphate group at Tyr15 is removed by phosphatases in the Cdc25 family; therefore, CDK activity can be influenced by the balance between inhibitory phosphorylation and activating dephosphorylation. There are three Cdc25 phosphatases (Cdc25A, Cdc25B and Cdc25C). Cdc25A acts at both G1/S and G2/M transition whereas Cdc25B and Cdc25C play roles in S and G2/M phases [7].

CDK regulation by the inhibitory phosphorylation has two biological implications. One is generation of a switch-like CDK activation mechanism. Wee1 and Cdc25 are both phosphorylated by CDKs, where Wee1 is inhibited while Cdc25 is activated by CDKs. Thus, there is a positive feedback mechanism built in the CDK activation: initial activation of a small fraction of CDK leads to activation of Cdc25 and inhibition of Wee1, tipping the balance between the inhibitory phosphorylation and the activating dephosphorylation. Another biological significance of the Tyr15 phosphorylation is to inhibit CDKs in the checkpoints in response to DNA damage (see Sect. 4.2).

CDK4 and CDK6 also contain a tyrosine residue equivalent to the Tyr15 residue of CDK2 and CDK1 (CDK4 Tyr17 and CDK6 Tyr24). It has been reported that TGF-β treatment causes CDK6 inhibition through increased Tyr24 phosphorylation via Cdc25A down-regulation. Similarly, ultraviolet radiation causes G1 arrest in a manner dependent on tyrosine phosphorylation of CDK4. The Tyr kinases responsible for these effects are unknown, but it is unlikely Wee1 because Wee1 lacks kinase activity toward cyclin D-associated CDKs in vitro.

CDK Inhibitors

The Cip/Kip Family

$p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ belong to the Cip/Kip family of CDK inhibitors, which mainly inhibit CDK4, CDK6 and CDK2. These inhibitors share a conserved N-terminal region where they interact with both CDK and cyclins. The crystal structure of $p27^{Kip1}$ bound to CDK2-cyclin A revealed several mechanisms of CDK inhibition by CDK inhibitors. First, $p27^{Kip1}$ binding with cyclin A blocks the interaction of substrates with cyclins. The same Cy motif (RXL motif) that is utilized in the substrate recognition by cyclins is also present in $p21^{Cip1}$, $p27^{Kip1}$, and

$p57^{Kip2}$, therefore preventing substrate interactions competitively. Second, $p27^{Kip1}$ inserts the inhibitory 3_{10} helix into the catalytic cleft of CDKs, preventing ATP binding. Third, in the case of CDK4/6, $p27^{Kip1}$ blocks the access of CAK to the T-loop, thereby inhibiting CDK activation indirectly.

Paradoxically, the Cip/Kip family proteins can assist complex formation of CDK4/6 and cyclin D under certain circumstances and promote cell-cycle entry. Mechanistically, mitogenic signals induce $p27^{Kip1}$ phosphorylation at Tyr88 (and weakly Tyr89) by tyrosine kinases and displace the inhibitory 3_{10} helix from the catalytic cleft. $p27^{Kip1}$ phosphorylation by Tyr kinases, therefore, neutralizes its inhibitory effects on CDK4/6 and converts $p27^{Kip1}$ to an assembly factor for CDK4/6-cyclin D. A similar mechanism has been proposed for $p21^{Cip1}$-dependent assembly of the CDK4-cyclin D complexes. Therefore, the Cip/Kip family proteins play opposite roles in quiescent and cycling cells: they inhibit residual CDK4/6 activities in G0 cells, while they switch to activators of CDK4/6 when mitogenic stimuli trigger tyrosine phosphorylation. In addition, the noninhibitory binding of $p21^{Cip1}$ and $p27^{Kip1}$ with CDK4/6-cyclin D sequesters the CDK inhibitors from CDK2-cyclin E/A, facilitating their activation following the cell-cycle entry.

Each Cip/Kip family inhibitor has its own regulatory mechanisms and plays distinct roles in the cell-cycle regulation. $p21^{Cip1}$ is induced at the transcriptional level by the tumor suppressor p53 in response to DNA damage (see Sect. 4.2.1). $p21^{Cip1}$ protein levels are regulated in the cell cycle as well. Phosphorylation of $p21^{Cip1}$ by CDK2-cyclin E at the G1/S transition induces its recognition and ubiquitination by the E3 ubiquitin ligase SCF^{Skp2} followed by proteasome-mediated degradation. $p21^{Cip1}$ also binds PCNA, a sliding clamp for DNA polymerases, through the PCNA-interacting peptide motif in the C-terminus, and inhibits DNA replication in G1 phase. Once cells enter S phase, $p21^{Cip1}$ is ubiquitinated on PCNA by the E3 ubiquitin ligase $CRL4^{CDT2}$ and degraded by the proteasome.

$p27^{Kip1}$ is regulated by transcription, protein stability, and cellular localization. In cycling cells, Thr187 phosphorylation of $p27^{Kip1}$ by CDK2-cyclin E induces recognition and ubiquitination by the E3 ubiquitin ligase SCF^{Skp2}, causing more activation of CDK2-cyclin E. This positive feedback mechanism contributes to the switch-like activation of CDK2-cyclin E at the G1/S transition. In quiescent cells, on the other hand, $p27^{Kip1}$ proteins accumulate in nuclei due to down-regulation of Skp2 and inhibit residual CDK activity. Upon cell-cycle entry, mitogenic stimuli promote $p27^{Kip1}$ inactivation by a number of mechanisms. First, Ser10 phosphorylation of $p27^{Kip1}$ by Akt and other kinases promotes nuclear export of $p27^{Kip1}$ in G1 phase by creating a binding site for exportin-1. Second, phosphorylation at Thr157 by Akt, and Thr198 by Akt and p90 ribosomal S6 kinase enhances retention of $p27^{Kip1}$ in cytoplasm by creating binding site for 14-3-3, which blocks nuclear import of $p27^{Kip1}$. Third, this pool of $p27^{Kip1}$ proteins in the cytosol is ubiquitinated by the E3 ubiquitin ligase KPC and degraded by the proteasome.

$p57^{Kip2}$ is mainly involved in embryogenesis and shows tissue-specific expression patterns. Its expression can be induced by epigenetic modulation to block pancreatic cancer proliferation [8] (see Sect. 6.2).

The INK4 Family

The INK4 family of CDK inhibitors consists of $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$. The family members share a similar domain structure of multiple ankylin repeats and function as inhibitors of CDK4 and CDK6. Unlike the Cip/Kip family members, INK4 proteins interact with monomeric CDKs. Structural studies of $p16^{INK4a}$ and $p19^{INK4d}$ bound to CDK6 suggest that INK4 proteins bind CDK6 opposite to the cyclin D binding site. Binding with the INK4 family inhibitors induces conformational changes that make CDK6 incompatible to cyclin D binding. It also distorts the catalytic cleft and interferes with ATP binding to the active site. INK4 family inhibitors also block the access of $p27^{Kip2}$ to CDK6, thereby causing redistribution of the Cip/Kip family inhibitors to CDK2.

Among the INK4 family members, $p16^{INK4a}$ and $p15^{INK4b}$ are clearly linked to tumor suppressor functions. $p16^{INK4a}$ can be induced by oncogenic RAS signaling, which leads to phosphorylation-dependent activation of the ETS family transcription factors. On the other hand, $p15^{INK4b}$ is induced by antimitogen TGF-β, through SMAD proteins.

Regulation of Cell-Cycle Entry

Signals from extracellular growth factors promote cell proliferation by driving cell-cycle entry at G0/G1 phase. The entry point of the mitogenic signals to the cell cycle is the promoters of *CCND1*, *CCND2,* and *CCND3*, which encode cyclin D1, D2, and D3, respectively. Each cyclin D gene is transcriptionally activated in response to different mitogenic stimuli. The key target of CDK4/6-cyclin D-mediated phosphorylation is the tumor suppressor protein pRB. pRB and its family members, p107 and p130, are repressors of the E2F transcription factors, which bind the promoters of genes that are necessary for S phase and M phase. Phosphorylation of pRB by CDK4/6-cyclin D induces dissociation of pRB from E2F and trigger transcriptional activation of downstream genes. Among the targets activated by E2F are the genes encoding cyclin E and cyclin A. CDK4/6-cyclin D activation in response to mitogenic stimulation, therefore, starts the autonomous signaling cascade of CDK activation, which becomes independent of growth stimuli after passing the restriction point (R-point) in G1 phase (Fig. 2).

E2F Transcription Factors

E2Fs are a family of transcription factors consisting of E2F1 to E2F8 [9]. E2F1 to E2F6 contain a single DNA-binding domain and form a dimer with DP1 or DP2 proteins to bind DNA. In contrast, E2F7 and E2F8 contain two DNA-binding domains and act without forming a heterodimer with DP1 and DP2. There is a third DP family protein, DP4, which has been shown to forms a dimer with E2F but lacks the DNA binding ability. E2F-DP dimers bind the consensus sequence, TTTCCCGC, with slight variations. A large number of genes that are involved in

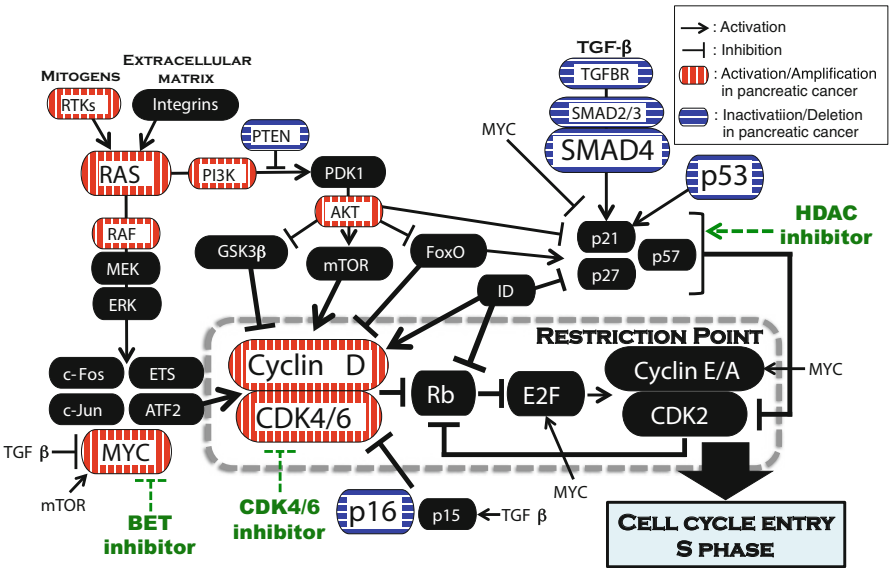

Fig. 2 Cell-cycle entry and its dysregulation. Mitogens interact with receptor tyrosine kinases (RTKs) and trigger RAS activation. RAS activates the RAF-MEK-ERK pathway, resulting in activation of transcription factors (c-Fos, c-Jun, ATF2, ETS, and MYC) to induce expression of cyclin D proteins. RAS also activates the PI3K-AKT pathway, which is antagonized by PTEN. Activated AKT regulates its effectors ($p21^{Cip1}$, $p27^{Kip1}$, GSK3β, FoxO, and mTOR) and promotes cell-cycle entry. ID proteins also promote cell-cycle entry by inhibiting bHLH/Zip transcription factors and pRb. Once cyclin D proteins are expressed, CDK4/6-cyclin D phosphorylates and inactivates pRb, resulting in derepression of E2F. Derepressed E2F induces expression of cyclin E, and CDK2-cyclin E phosphorylates pRb further to induce cyclin A expression for S phase. In various conditions, CDK inhibitors ($p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$, $p16^{INK4a}$, and $p15^{INK4b}$) are induced to stop the cell cycle. The canonical TGF-β signaling pathway can stop the cell cycle at G1 phase through down-regulation of MYC and induction of CDK inhibitors ($p15^{INK4b}$ and $p21^{Cip1}$). Frequent mutations observed in pancreatic cancer are indicated by vertical stripes (activation or amplification) and horizontal stripes (inactivation and deletion). *KRAS* (>95%), *CDKN2A* (>80%), *TP53* (>80%), and *SMAD4* (55%) are among the most common mutations. In addition, genetic alterations (mutations, deletions, and amplifications) are found in genes encoding RTKs (*MET* and *ERBB2*), proliferation signaling components (*MYC*, *AKT2*, *PTEN*, *PI3KCA,* and *PI3KR3*), CDK-cyclins (*CCND1*, *CDK6,* and *CDK4*), and TGF-β signaling components (*TGFBR1*, *TGFBR2,* and *SMAD3*). Potential therapeutic drugs (CDK4/6 inhibitors, HDAC inhibitors, and BET inhibitors) are also indicated

G1/S transition, DNA replication and mitosis carry the E2F binding motifs at their promoter and are coregulated during the cell cycle.

E2F1 to E2F3 are transcriptional activators and their transactivation can be repressed by pRB binding. In contrast, E2F4 and E2F5 are generally considered transcriptional repressors and they operate in cooperation with p107 and p130. E2F6 to E2F8 are also transcriptional repressors, but they repress transcription independently of pRB family proteins.

Regulation of E2F by pRB Family Proteins

pRB family proteins, pRB, p107, and p130, are transcriptional repressors of E2F transcription factors [10] (Fig. 2). In G0 phase, repressor E2Fs (E2F4 and E2F5) in conjunction with p107 or p130 repress E2F target genes, which include genes encoding activator E2Fs. Upon cell-cycle entry, E2F4 is removed from the nuclei through active nuclear export. Activator E2Fs (E2F1-E2F3) in turn occupy the E2F binding sequences; however, they are repressed by pRB until CDK4/6-cyclin D becomes active.

Several mechanisms contribute to the repression of E2Fs by the pRB family members [11]. Recruitment of a histone deacetylases (HDAC) by pRB family proteins reduces histone acetylation levels and makes chromatin more closed. In addition, the ATP-dependent chromatin remodeling complex, SWI/SNF, remodels chromatin into repressed state. Binding with pRB also physically blocks the access of transcriptional activators to E2Fs.

When CDK4/6-cyclin D is activated in G1 phase, it phosphorylates pRB and releases HDAC from pRB, relieving the repression of the Cyclin E gene at the G1/S transition. SWI/SNF remains on pRB and it is sufficient to suppress the cyclin A gene. pRB phosphorylation by CDK4/6-cyclin D also derepresses activator E2Fs at the promoter of the *E2F1* gene itself, thereby accelerating E2F1 production through a positive feedback. Next, activated CDK2-cyclin E phosphorylates additional sites on pRB and relieves E2Fs from pRB-mediated repression at the cyclin A gene. Activated CDK2-cyclin A in turn maintains pRB phosphorylation during S phase.

Activation of CDK2-cyclin A also initiates a program that down-regulates E2F transactivation. CDK2-cyclin A interacts with activator E2Fs and phosphorylates the DP subunit. This phosphorylation inhibits DNA binding of the E2F1-DP1 dimer. In addition, E2F1 is ubiquitinated by SCF^{Skp2} and degraded by the proteasome in S phase.

DNA Damage Response

Genome integrity is constantly threatened by both extrinsic and intrinsic sources of DNA damage. Although DNA damage is normally repaired by specific mechanisms, unrepaired DNA damage can cause mutations and genome instability, which are hallmarks of cancer. To avoid such adverse consequences, cells have evolved a network of cellular processes called DNA-damage response (DDR). Double-strand DNA breaks (DSBs) directly activate DDR, while other types of damages, such as base loss or modifications, do not activate DDR until they stall DNA replication forks. DNA replication, therefore, works as a sensor for DDR activation for many types of DNA damage. DDR has two major functions: (1) cell-cycle arrest through a process called cell-cycle checkpoint and (2) activation of DSB repair or stabilization of stalled replication forks. If the damage is irreparable, DDR signals cells to undergo senescence (permanent cell-cycle arrest) or programmed cell death (apoptosis). Defects in the DDR pathways have been associated with various

diseases and cancer-predisposition syndromes, illustrating its significance in human health [12].

DNA Damage Signaling

DDR pathways comprise a multitude of proteins that detect DNA damage and transduce the signals to downstream effectors. These effectors execute cellular responses such as cell-cycle arrest, DNA repair, and/or apoptosis. The key upstream components in the DNA damage signaling cascades are the serine/threonine protein kinases in the phosphatidylinositol-3-kinase related kinase (PIKK) family: ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia and Rad3-related (ATR). ATM and ATR phosphorylate numerous proteins, working as the master transducers of DDR signaling. ATM responds primarily to DSBs, while ATR can be activated by single-stranded DNA (ssDNA) that is indirectly generated after DNA replication is stalled at the DNA lesions.

ATM-Mediated Signaling in Response to DSBs

DSBs are first recognized by the sensor called Mre11-Rad50-Nbs1 (MRN) complexes. This promotes rapid localization and activation of the transducer kinase ATM, which responds to DSBs throughout the cell cycle. One well-known target of ATM is the histone variant H2AX, which is phosphorylated at Ser139 by ATM in response to DSBs. The phosphorylation of H2AX (**γ**H2AX) creates a direct binding site for MDC1, which in turn amplifies the local ATM signaling, leading to spreading of activated ATM and **γ**H2AX along chromatin. This amplification of ATM activity in turn promotes the recruitment and retention of additional mediator proteins such as BRCA1 and 53BP1 at the damage sites, which further promote recruitment of other repair factors.

ATR-Mediated Signaling in Response to Single-Stranded DNA

Many types of DNA damage activate DDR indirectly by blocking DNA replication. Replication-blocking lesions generate ssDNA at stalled replication forks because DNA helicase uncoupled from DNA synthesis continues to unwind double-stranded DNA (dsDNA). In addition, ssDNA can also be generated from DSBs following its end resection during the DSB repair in S and G2 phases (see Sects. 4.2.2 and 4.3.2). ssDNA is a rather unusual structure in cells and generates a signal for ATR-dependent DDR activation.

Cell-Cycle Checkpoints

To stop the cell cycle in response to DDR activation, cells rely on three major cell-cycle checkpoints: G1/S, intra-S, and G2/M (Fig. 1). These checkpoints block the cell cycle by inhibiting CDK, the driving force of the cell cycle.

G1/S Checkpoint

When DSBs occur during G1 phase, the G1/S checkpoint is activated to prevent cells from entering into S phase. The ATM kinase activated by DSBs initiates the G1/S checkpoint by phosphorylating the downstream kinase Chk2 at Thr68. Activated Chk2 in turn phosphorylates Cdc25A, a phosphatase that removes inhibitory phosphorylation of CDK2, causing its ubiquitin-dependent degradation (see Sect. 2.3). As a result, activation of CDK2-cyclin E and CDK2-cyclin A is blocked and cells arrest in G1 phase.

The G1/S checkpoint is also highly dependent on the p53 protein, a tumor suppressor that functions primarily as a transcription factor and plays a role in cell-cycle arrest and apoptosis. In response to DSBs, ATM, and Chk2 phosphorylate p53 and prevent the binding with its negative regulator Mdm2, the ubiquitin ligase that promotes p53 degradation, thereby leading to p53 stabilization and activation. This in turn promotes the transcriptional activation of p53-target genes, including the CDK inhibitor $p21^{Cip1}$, which inhibits CDK2 and prevents S-phase entry.

Intra-S Checkpoint

The intra-S checkpoint can be activated either by DSBs or stalled DNA replication. In the case of DSBs, activated ATM promotes resection of the 5′ end of DSBs for homologous recombination (HR), which is a preferred DSB repair pathway in S and G2 phases (see Sect. 4.3.2). The resection of the 5′ DNA ends creates stretches of ssDNA and activates the ATR-mediated checkpoint, enabling transition of the DDR signaling from ATM to ATR. Stalled DNA replication, on the other hand, results in the formation of ssDNA due to uncoupling between the replicative helicase and the replicative polymerase.

The exposed ssDNA is rapidly coated with ssDNA-binding protein RPA. RPA-bound ssDNA recruits the ATR-ATRIP complex and promotes ATR autophosphorylation *in trans* at Thr1989 [13, 14]. RPA-bound ssDNA also signals for the recruitment of the Rad17-RFC2-5 clamp loader at the junction of RPA-ssDNA and dsDNA, which in turn loads the heterotrimeric ring-shaped Rad9, Hus1, and Rad1 (9-1-1) complex onto chromatin. The phosphorylation of Rad9 in the 9-1-1 complex facilitates the recruitment of TopBP1, which then interacts with ATR-ATRIP complex on RPA-coated ssDNA through the Thr1989 phosphorylation site on ATR. This interaction between TopBP1 and ATR-ATRIP further stimulates ATR kinase activity and/or facilitates its substrate recognition [15, 16].

Once fully activated, ATR phosphorylates and activates its downstream effector kinase Chk1 on Ser317 and Ser345 with the help of several mediator proteins such as Claspin and Tim/Tipin. Activated Chk1 transduces the signal for intra-S-phase arrest by preventing late origin firing or replication initiation until the damage is repaired [17]. Mechanistically, Chk1-mediated phosphorylation of Cdc25A promotes its proteasomal degradation, leading to CDK2 inactivation. Chk1 may also inhibit the Cdc7-Dbf4 kinase activity [18, 19]. Since CDK2 and Cdc7-Dbf4 are necessary for activation of the replicative helicase, inhibition of the two kinases block further origin firing, causing intra-S-phase arrest [17].

G2/M Checkpoint

G2/M checkpoint is activated to prevent cells with damaged DNA or incompletely replicated DNA from entering into mitosis, while providing time to repair the damage. Under normal circumstances, the CDK1-cyclin B complex promotes the entry into mitosis and therefore is the critical target of the G2/M checkpoint. In the presence of DNA damage in G2 phase, activated ATR and Chk1 kinases phosphorylate Cdc25A or Cdc25C, causing their inactivation by ubiquitin-mediated proteolysis (Cdc25A) or cytoplasmic sequestration through a binding to 14-3-3 proteins (Cdc25C) [20]. Since these phosphatases are necessary for CDK1-cyclin B activation through the removal of inhibitory Tyr15 phosphorylation on CDK1 (see Sect. 2.3), inactivation of Cdc25A and Cdc25C by the checkpoint leads to cell-cycle arrest in G2 phase. ATM-Chk2 also plays an important role in the initiation of the G2 arrest after DSBs, although ATR/Chk1 mediated signaling are required for the maintenance of G2/M checkpoint [21].

The G2/M arrest is also maintained though stabilization of the Wee1 kinase induced by Chk1-mediated phosphorylation [20, 22]. Wee1 stabilization enhances the inhibitory Tyr15 phosphorylation on CDK1 and promotes its inactivation (see Sect. 2.3).

G2/M checkpoint is the last resort for cells to ensure that damaged DNA or under-replicated DNA is not carried over to mitosis. If the cells containing unrepaired DNA or under-replicated DNA are forced to progress into mitosis, it leads to mitotic catastrophe, a mechanism that causes cells to die either in M phase or undergo cell death or senescence in the subsequent G1 phase [23].

DNA Repair

DNA Repair Pathways

Besides signaling for the cell-cycle arrest, ATM/ATR kinases also signal for efficient repair of damaged DNA through phosphorylation and recruitment of multiple repair factors to the damage sites. Given the diversity of DNA lesions, cells have evolved different types of lesion specific repair pathways, which are briefly described below.

- Base excision repair (BER): BER repairs small chemical changes or nonhelix distorting adducts of DNA bases such as oxidized bases through removal of the damaged base. DNA single-strand breaks (SSBs) that can arise from direct attack by reactive oxygen species or other reactive metabolites are repaired by the BER pathway with an involvement of a key enzyme called PARP-1 (see Sect. 6.3).
- Nucleotide excision repair (NER): More complex or helix distorting bulky lesions such as intrastrand crosslinks (covalent crosslinks between bases on the same DNA strand) are repaired by NER. NER involves excision of oligonucleotides containing the damaged bases.

- Mismatch repair (MMR): MMR repairs mispaired or misincorporated nucleotides arising from errors of DNA polymerases by replacing them with correct nucleotides.
- Fanconi anemia (FA) pathway: The FA pathway repairs DNA interstrand crosslinks (covalent crosslinks between the two DNA strands) that can be generated by chemotherapeutic agents as well as by endogenous metabolic products. The FA pathway involves cooperation of different repair pathways, including NER, HR, and translesion synthesis.
- Homologous recombination (HR) and nonhomologous end joining (NHEJ): HR or NHEJ repairs DSBs that can arise from exposure to ionizing radiation (IR), chemotherapeutic drugs or intrinsic fork collapse.

This chapter will focus on DSBs repair mechanisms because of the deficiencies in this pathway in some pancreatic cancer.

DNA Double-Strand Break Repair

DSBs are toxic DNA lesions as it can result in extensive loss of chromosomal content, gross chromosomal rearrangements, and/or mutations, leading to cell death or cancer. Human cells rely on two major mechanisms for DSBs repair: NHEJ and HR (Fig. 3). NHEJ is the predominant DSB repair pathway in G0/G1 phase, while HR is used in S and G2 phases, in which sister chromatids are available as a recombination template.

NHEJ NHEJ favors direct ligation of broken DNA ends. NHEJ begins with binding of the Ku70-Ku80 heterodimer to the broken DNA ends. Ku70-Ku80 serves as a scaffold for recruiting and activating DNA-PKcs, a PIKK family kinase. This further recruits end-processing factors such as the nuclease Artemis, which trims and prepares DNA ends for ligation by the DNA ligase IV/XRCC4/XLF complex. Although NHEJ is highly efficient, they are error-prone due to the trimming-ligation approach of this process. DSBs are channeled to NHEJ in G1 phase by 53BP1. 53BP1 not only promotes the recruitment of NHEJ-promoting factor RIF1 but also inhibits the accumulation of HR-promoting protein, BRCA1, to DSB sites [24].

HR HR, unlike NHEJ, is a high-fidelity process as it uses a homologous sister chromatid as a template to identically replace the genetic information on the broken DNA. HR occurs primarily in S and G2 phases as the sister chromatid is readily available as a repair template. HR repair is initiated with the resection of the 5′ ends of DSBs by several nucleases in a BRCA1-dependent manner. The MRN complex and the CtIP nuclease initiate limited resection of the 5′ broken ends. This is followed by extensive resection by EXO1 and BLM-DNA2 that results in the formation of longer stretches of 3′ ssDNA on either side of the DSB. DSBs are channeled to HR rather than NHEJ by BRCA1, which not only promotes efficient 5′ end resection required for HR but also inhibits the recruitment of NHEJ-promoting RIF1 to DSB sites [24].

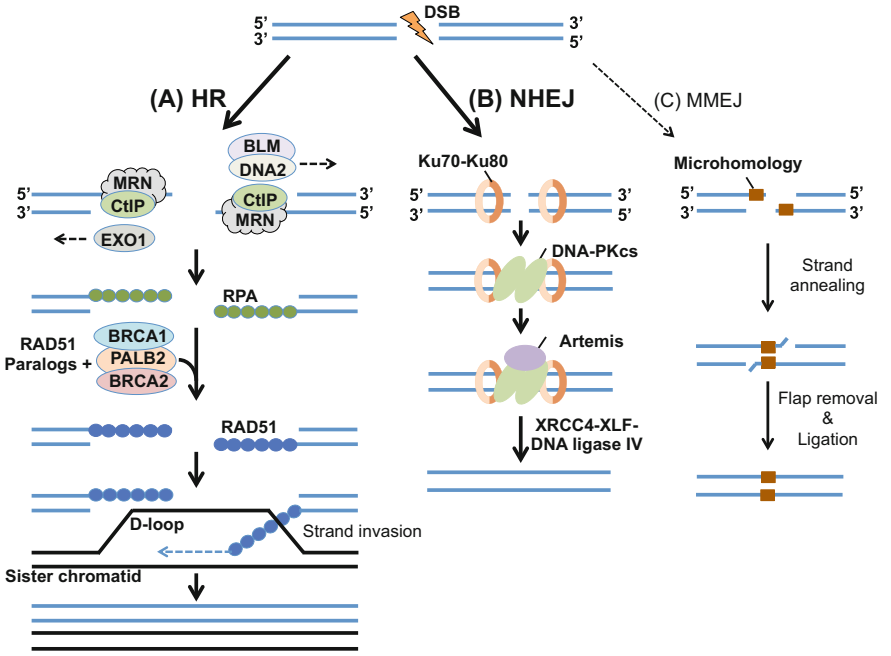

Fig. 3 Double-strand DNA breaks repair pathways. Double-strand DNA breaks (DSBs) are repaired predominantly by (**a**) homologous recombination (HR) or (**b**) error-prone nonhomologous end joining (NHEJ). (**a**) HR initiates with MRN-CtIP complex-mediated 5′ end resection of the broken DNA ends, followed by extensive resection by EXO1 and BLM-DNA2 nucleases. The resulting long stretch of 3′ single-strand DNA (ssDNA) is coated with RPA, which is next displaced by Rad51 in a process dependent on BRCA1-PALB2-BRCA2 and Rad51 paralogs. The Rad51-ssDNA filament performs homologous sequence search and promotes strand invasion into an undamaged sister chromatid, forming a displacement loop (D-loop). Following DNA synthesis at the resected strand, the resulting intermediate structures are resolved to complete the repair. (**b**) NHEJ initiates with the binding of Ku70–80 to the broken DNA, where it prevents end resection. This in turn promotes recruitment of several downstream factors including DNA-PKcs and Artemis, which process the broken DNA ends for ligation by the XRCC4-XLF-DNA ligase IV complex. (**c**) DSBs can also be repaired by alternative-NHEJ, also known as microhomology-mediated end joining (MMEJ), in a less-efficient manner. MMEJ initiates with short resection of the broken DNA, where it exposes microhomology sequences near the broken DNA ends. After the microhomology sequences at the ssDNA are annealed, the flaps are removed and the DNA ends are ligated

The stretches of ssDNA formed after resections are rapidly coated by RPA to inhibit the formation of secondary DNA structures and to facilitate the loading of the recombinase Rad51. The formation of RPA-coated ssDNA also activates ATR-mediated signaling (see Sect. 4.2.2). RPA is then displaced by Rad51 to generate Rad51-ssDNA nucleoprotein filament in a manner dependent on BRCA1, PALB2, BRCA2, and Rad51 paralogs. In particular, BRCA1 promotes the recruitment of BRCA2 to DSBs through the interaction with PALB2, which acts a bridge between BRCA1 and BRCA2. The BRCA1-PALB2-BRCA2 complex together with

Rad51 paralogs assists in the loading of Rad51 onto ssDNA to generate the Rad51-ssDNA recombinase filament. This filament promotes homology search and strand invasion into an undamaged homologous duplex or sister chromatid through formation of a displacement loop (D-loop). DNA synthesis is carried out at the resected strand using the undamaged homologous strand as a template. The resulting intermediate structure formed as a consequence is resolved by resolvases to complete the repair process.

Alternative NHEJ Although regarded more as a less-efficient back up process, DSBs can also be repaired by alternative NHEJ, also known as microhomology-mediated end joining (MMEJ) [25] (Fig. 3). This process involves joining of DSBs using microhomologous sequences flanking the DSB to align the ends for repair. MMEJ causes deletions at the break sites and therefore is a mutagenic repair process.

Cell-Cycle Alterations in Pancreatic Cancer

Cell-cycle entry is tightly regulated and normal cells proliferate only when stimulated by mitogenic signals. In cancer cells, however, cell-cycle entry is constantly driven by dysregulated mitogenic signals, resulting in aberrant proliferation, which is a hallmark of cancer. Recent studies using whole genome sequencing have revealed frequent mutations that influence cell-cycle entry in pancreatic cancer cells [26–30]. Among the most commonly mutated genes are *KRAS*, *CDKN2A*, *TP53,* and *SMAD4*. In general, *KRAS* mutations are observed in early lesions (PanIN-1) followed by *CDKN2A* mutations (PanIN-2), whereas *TP53* and *SMAD4* mutations (PanIN-3) drive tumorigenesis further, eventually resulting in advanced pancreatic cancer. This section will describe how major mutations in pancreatic cancer dysregulate cell-cycle entry, and how they lead to aberrant proliferation in pancreatic cancer.

Constitutive Activation of KRAS

Proliferation signaling pathways play a crucial role in tissue homeostasis; however, their overactivation can cause uncontrolled proliferation in cancer. Activating mutations in the *KRAS* gene are seen in most pancreatic cancers (>95%), in which substitution of glycine 12 (G12D or G12 V) is the major type of mutation. Activity of RAS proteins, which belong to the small GTPase family, is regulated by the binding of guanosine triphosphate (GTP) or guanosine diphosphate (GDP). When cells receive mitogenic signals from receptor tyrosine kinases (RTKs) and integrins, RAS proteins become an active form (RAS-GTP), which binds to its effectors and activates the downstream pathways (Fig. 2). Wild-type RAS proteins hydrolyze GTP to GDP by intrinsic GTPase activity, and this leads to inactivation of RAS proteins (RAS-GDP). Mutations in *RAS* genes usually impair this GTPase activity. Due to the

lack of GTPase activity, mutant RAS is unable to terminate the active state, thereby activating its downstream targets constitutively.

The RAF-MEK-ERK pathway is one of the major downstream pathways of RAS and strongly promotes the cell-cycle entry. RAF, MEK, and ERK are kinases, and they transmit the proliferation signal by phosphorylation. Once active RAS interacts with its effector RAF, RAF phosphorylates and activates MEK. Activated MEK also phosphorylates and activates ERK. Eventually, activated ERK can induce cyclin D1 and cyclin D2 through the activation of transcription factors, such as AP-1 family (c-FOS, c-JUN, ATF2), ETS family, and MYC transcription factors.

The PI3K-AKT pathway is another important downstream pathway of RAS to promote the cell-cycle entry. RAS activates the lipid kinase PI3K, which converts phosphatidylinositol diphosphate (PIP2) to phosphatidylinositol triphosphate (PIP3). This reaction can be reversed by a tumor suppressor protein PTEN. PIP3 provides a docking site for the kinases, AKT and PDK1, at the cellular membrane, where PDK1 activates AKT. Activated AKT promotes cell-cycle entry by several mechanisms. First, AKT-mediated phosphorylation inactivates CDK inhibitors $p21^{Cip1}$ and $p27^{Kip1}$. Phosphorylation of $p21^{Cip1}$ by AKT inhibits its activity to bind CDK2 as well as PCNA, while phosphorylation of $p27^{Kip1}$ causes its cytoplasmic accumulation (see Sect. 2.4.1). Second, AKT phosphorylates GSK3β and inhibits its activity. This results in stabilization of cyclin D1, which is targeted for degradation upon phosphorylation by GSK3β. Third, AKT inhibits FOXO family transcription factors by inhibitory phosphorylation. This leads to changes in FOXO-regulated genes: down-regulation of $p27^{Kip1}$ and up-regulation of cyclin D1 and D2. Lastly, AKT activates the mTOR pathway. The mTOR pathway increases Cyclin D1 and MYC protein synthesis and thereby promotes cell-cycle entry. Taken together, constitutively active KRAS enhances proliferation signals and promotes cell-cycle entry by up-regulating cyclin D and inhibiting CDK inhibitors.

Inactivation of the TGF-β Signaling

Transforming growth factor-β (TGF-β) regulates a wide variety of biological processes including cell proliferation, differentiation, migration, and apoptosis. Binding of TGF-β to the receptors (TGFBR1/2) causes phosphorylation of the transcription factor SMAD2 or SMAD3 by the serine/threonine kinase in the intracellular domain of the receptors. This phosphorylation triggers oligomerization of SMAD2/3 with SMAD4 and its translocation to the nucleus, where it regulates their target gene expression with cofactors. The effects of TGF-β can be either tumor-suppressive or oncogenic. TGF-β induces cell-cycle arrest and apoptosis (tumor-suppressive), while it promotes epithelial-mesenchymal transition (EMT) and changes tumor microenvironment (oncogenic). The SMAD-dependent canonical signaling induces cell-cycle arrest in G1 phase through down-regulation of MYC and induction of CDK inhibitors $p15^{INK4b}$ and $p21^{Cip1}$ (Fig. 2). Numerous studies have found that the canonical TGF-β signaling is impaired in several types of cancers. In pancreatic cancer, inactivating mutations are found in the genes encoding SMAD4 (55%),

TGFBR1/2 (5–10%), and SMAD3 (<5%) [30]. These mutations render cancer cells insensitive to the cytostatic effects of TGF-β. Taken together, inactivation of the canonical TGF-β signaling allows pancreatic cancer cells escape from the tumor suppressive effects of TGF-β, while they exploit its oncogenic power.

Inactivation of $p16^{INK4a}$

More than 80% of pancreatic cancers exhibit aberrations in the *CDKN2A* locus [31]. The *CDKN2A* gene is usually inactivated by homozygous deletions or loss of heterozygosity (LOH) involving point mutations that result in frame shifts, premature stop codons or aberrant splicing. In addition, gene silencing by promoter methylation was also observed and, if these are included, nearly 100% of the *CDKN2A* genes may be inactivated in pancreatic cancer [32].

CDKN2A encodes two different proteins, $p16^{INK4a}$ and $p14^{Arf}$, which are important for the cell cycle and apoptosis. $p16^{INK4a}$ binds to CDK4/6 and inhibits its activity (see Sect. 2.4.2 and Fig. 2) while $p14^{Arf}$ binds to MDM2, an E3 ubiquitin ligase for p53, and stabilizes p53. Interestingly, mutations in pancreatic cancer tend to specifically target $p16^{INK4a}$, although both $p16^{INK4a}$ and $p14^{Arf}$ can be inactivated. This indicates a specific role of $p16^{INK4a}$ inactivation in the development of pancreatic cancer, strongly suggesting that elevated CDK4/6-cyclin D activity is an important driver of pancreatic cancer. Consistent with this notion, amplification of genes encoding cyclin D1 (9%), CDK4 (6%), and CDK6 (7%) are also found in pancreatic cancer [26, 28].

Overexpression of MYC

MYC proto-oncogene encodes a basic helix-loop-helix/leucine zipper (bHLH/Zip) family transcription factor, and its function is associated with many biological processes such as cell cycle, metabolism, and cell stemness. Up-regulation of *MYC* is often seen in many types of cancer including pancreatic cancer, and it contributes to a variety of cancer phenotypes including increased cell proliferation, cancer stem cells, drug resistance, and metastasis. In pancreatic cancer, MYC is often overexpressed by gene amplification (15–30%) and other mechanisms such as activation of RTK-RAS-ERK, WNT/β-catenin signaling, and inactivation of TGF-β signaling [26, 33]. It was recently shown that the squamous type pancreatic cancer, which is characterized by MYC pathway activation, is strongly associated with poor prognosis [29]. MYC can strongly promote the cell cycle by activating and suppressing transcription of target genes (Fig. 2). MYC activates genes encoding cell-cycle positive regulators such as cyclin D2, cyclin E1, CDK4, Cdc25A, and E2F1, while repressing negative regulators including CDK inhibitors ($p21^{Cip1}$ and $p27^{Kip1}$) and miRNAs that target critical cell-cycle regulators (let-7 family, miR-15a/16-1, miR-26a, and miR-34a). Taken together, overexpression of MYC drives cell-cycle progression by regulating its target genes related to the cell cycle.

Overexpression of ID Proteins

Inhibitors of DNA binding (ID) proteins (ID1-3) are often overexpressed in pancreatic cancer [34–36]. ID proteins form a heterodimer with bHLH transcription factors and inhibit its DNA binding. In general, bHLH transcription factors can induce cell differentiation and the cell-cycle arrest, therefore, the elevated ID proteins is associated with the dedifferentiation and the high cell proliferation in cancer. Mechanistically, inhibition of bHLH transcription factors by overexpressed ID proteins results in down-regulation of cell-cycle inhibitors such as $p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$, and $p16^{INK4}$ and up-regulation of cyclin D1 and cyclin E (Fig. 2). Indeed, it was shown that the ID3 overexpression was sufficient to induce cell-cycle entry in human pancreatic cells [36]. Besides bHLH transcription factors, ID2 can directly bind pRB proteins and promote proliferation. The mechanism of ID overexpression in pancreatic cancer is not completely understood. Because many oncogenic pathways such as MYC, RAS, and Notch can induce ID proteins, it is possible that ID protein overexpression might be a consequence of other oncogenic events.

Inactivation of p53

The *TP53* gene is altered in more than 80% of pancreatic cancers [37]. Missense mutations in the DNA binding domain are observed most frequently, while other mutations such as frame shift mutations and deletions, complete absence of the protein without any mutations are also found. p53 protects cells from oncogenic stresses, such as DNA damage and impairment of ribosome biogenesis. Once p53 is activated by these stresses, it induces target gene expression as a transcription factor and causes cell-cycle arrest and apoptosis depending on the degree of the stress. The most well-studied target of p53 is the CDK inhibitor $p21^{Cip1}$, which inhibits CDK2-cyclin E activity and causes G1 arrest as a part of the G1/S checkpoint mechanism (see Sect. 4.2.1 and Figs. 1 and 2). Defective p53 allows cell-cycle entry even under stress conditions, thereby disrupting the barrier to tumorigenesis.

However, evidence is also accumulating that some of the mutant p53 proteins found in cancer might be more than just inactive p53: they instead acquire new functions (gain-of-function). For example, p53 with point mutations in the DNA binding domain, such as R175H and R273H, exhibit aberrant transcriptional activity. In response to DNA damage, mutant p53 proteins recruit histone acetyltransferase to the promoters of the genes for cyclin A2, cyclin B1, CDK1, and Cdc25C for their activation, while wild-type p53 recruits histone deacetylase HDAC1 for repression [38]. Gain-of-function of mutant p53 has also been demonstrated using pancreatic cancer mouse models. Mutant p53 causes dysregulation of genes related to proliferation and cell migration, and it is necessary for the metastatic phenotype of pancreatic cancer [39, 40]. In summary, mutant p53 proteins lose its canonical functions in the cell-cycle checkpoint, while some of the point mutants may gain new functions that promote pancreatic cancer.

Mutations in the BRCA Pathway

Germline mutations in the BRCA pathway genes, *BRCA1, BRCA2,* and *PALB2* (encoding BRCA2-interacting protein essential for HR), have been associated with increased susceptibility to pancreatic cancer [41–44] (Fig. 3). In addition, somatic mutations in *BRCA1* and *BRCA2* have been identified in pancreatic ductal adenocarcinoma (PDAC). These mutations are particularly associated with a subtype of PDAC (14%) that is characterized by a large number of abnormal chromosome structures such as duplication, deletions, and inversions and marked genome instability [28]. The majority of the tumors in this PDAC subtype exhibit characteristic mutational signatures, which is characterized by equal representation of all possible base substitution. These tumors also have large deletions harboring microhomology at breakpoint junctions, indicating that alternative NHEJ, rather than error-free HR, was used to repair DSBs. This signature is also found in ovarian and breast cancer with *BRCA1* and *BRCA2* mutations [28, 45].

Recent studies using genetically engineered mouse models provided insights in the roles of BRCA mutations in the PDAC development. The BRCA1 protein comprises multiple domains, including the N-terminal RING domain important for its E3 ligase activity and two BRCA C-terminal (BRCT) domains that mediate protein–protein interactions with other DDR proteins. Shakya et al. 2011 found that the mutations in the BRCT domain of BRCA1 accelerate oncogenic Kras-driven PDAC, whereas the mutation affecting the E3 ligase activity of BRCA1 does not affect PDAC pathogenesis [46]. This suggests the importance of BRCT domains, which are vital for its HR repair function, in BRCA1-mediated suppression of PDAC formation.

In accordance with the importance of the BRCA pathway in PDAC suppression, *Brca2* inactivation also promotes oncogenic Kras-driven PDAC in a mouse model [47]. Interestingly, contrary to the current view that loss of heterozygosity (somatic deletion of the wild-type allele) is required to stimulate the tissue-specific cancer in carriers with inherited heterozygous *BRCA2* mutations, germline heterozygous *Brca2* mutation was enough in promoting Kras-driven PDAC in this mouse model [47]. These tumors retained the wild-type *Brca2* allele and tumor-derived cell lines exhibited partial HR function [47]. These results suggest that *Brca2* may act as a haplo-insufficient tumor suppressor gene in the case of Kras-induced PDAC [48].

Therapeutic Opportunities

Pancreatic cancer has a poor prognosis with 5-year survival of around 5%. Standard initial chemotherapies for this disease have been antimetabolites, such as 5-fluorouracil (5-FU) and gemcitabine. Active metabolites of these drugs inhibit nucleoside synthesis and/or DNA replication, causing DNA damage and eventually cell death. Combination therapies such as FOLFIRINOX (5-FU, folinic acid, irinotecan, and oxaliplatin) and nab-paclitaxel plus gemcitabine are now first-line therapies for patients with metastatic pancreatic cancer and have shown improved

patient survival [49–52]. However, these drugs simply target highly proliferating cells, and they do not necessarily target the abnormal proliferation signals driving tumor formation. A number of new drugs have been proposed based on the features of pancreatic cancer including its genetic background and dysregulated pathways as described in the previous section. This section will introduce potential drug targets related to the cell cycle and the DNA repair pathways.

Inhibition of CDK4/6

As described in the previous section, major mutations and other dysregulated pathways in pancreatic cancer promote cell-cycle entry through up-regulation of cyclin D and the loss of $p16^{INK4a}$. Direct inhibition of CDK4/6-cyclin D is, therefore, an attractive strategy to stop aberrant proliferation of pancreatic cancer [53, 54] (Fig. 2). PD-0332991 (also known as Palbociclib) is a highly specific and orally-available CDK4/6 inhibitor. As expected, *CDKN2A* (encoding the CDK4/6 inhibitor $p16^{INK4a}$) mutant cancer cells are more sensitive to PD-0332991 as shown in a large scale drug sensitivity screen [55]. This drug, however, requires intact pRB, which represses E2F activity and stops the cell cycle upon CDK4/6 inhibition. Taking these points into consideration, pancreatic cancer might be sensitive to CDK4/6 inhibitors, because most of pancreatic cancers have intact pRB and inactivated *CDKN2A*.

Several groups, however, reported that a single-agent treatment of pancreatic cancer cell lines with PD-0332991 often showed a modest effect and development of resistance despite the initial response [56–58]. It turned out that the PI3K-AKT-mTOR pathway was up-regulated after the CDK4/6 inhibition, and expression of cyclin D1 and cyclin E1 were elevated consequently [57, 59]. Therefore, it appears that cancer cells overcame the CDK4/6 inhibition by enhanced proliferation signals and G1 cyclin overexpression. This raises a possibility of using a combination of drugs that inhibit CDK4/6 and bypassing pathways. Indeed, it was reported that inhibition of the PI3K-AKT-mTOR pathway by PI3K/mTOR inhibitors or insulin-like growth factor 1 receptor (IGF1R) inhibitor (IGF1R is one of the upstream RTKs of PI3K-AKT-mTOR pathway), or inhibition of RAF-MEK-ERK pathway by MEK1/2 inhibitor, synergized with the CDK4/6 inhibitor in vitro and xenograft models [56, 57]. Additional CDK4/6 inhibitors are in the pipeline (LY-2835219, abemaciclib; LEE011, ribociclib), and combination therapies of LY-2835219 and a PI3K/mTOR dual inhibitor are in phase II clinical trials for advanced pancreatic cancer. Thus, CDK4/6 inhibitors combined with other agents may be promising mechanism-based therapies on the horizon for pancreatic cancer.

Cell-Cycle Inhibition by Epigenetic Drugs

Aberrant transcription plays a major role in cancer. Epigenetic drugs, which inhibit writers, erasers, and readers of histone modifications, have been explored as therapeutic agents against pancreatic cancer [60]. While epigenetic drugs generally have

pleiotropic effects on cancer cells, induction of cell-cycle arrest is one of the key tumor suppression mechanisms. In pancreatic cancer, a number of studies have shown that treatment with histone deacetylase (HDAC) inhibitors induces cell growth arrest accompanied by up-regulation of the CDK inhibitors ($p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$, and $p19^{INK4d}$ [61]) (Fig. 2). Given that genes encoding CDK inhibitors are often silenced by aberrant histone deacetylation in cancer, derepression of CDK inhibitors by HDAC inhibitors might be a viable strategy to block cell proliferation.

A bromodomain and extraterminal (BET) family member protein BRD4 is another epigenetic factor that can be targeted therapeutically (Fig. 2). BRD4 binds acetylated histones via its BET domains at super enhancers and up-regulate transcription by promoting transcriptional elongation. BET inhibitors block the interaction of BRD4 with acetylated histones, thereby inhibiting transactivation of the target genes. A study using patient-xenograft model of pancreatic cancer showed inhibition of tumor growth by the BET inhibitor JQ1, accompanied by marked reduction of *CDC25B* expression [62]. In another study, a combinational treatment with JQ1 and the HDAC inhibitor SAHA synergistically suppressed tumor growth through induction of cell-cycle arrest and apoptosis in pancreatic cancer mouse models [8]. Suggested mechanisms of tumor suppression by JQ1 plus SAHA include *MYC* down-regulation and $p57^{Kip2}$ induction. Although the exact mechanism of the synergy between BET and HDAC inhibitors is not clear, combination therapies of epigenetic drugs could lead to improved treatment for pancreatic cancer.

PARP Inhibitors for BRCA Mutant Pancreatic Cancer

PARP (poly [ADP-ribose] polymerase) is an enzyme that is best known for its role in DNA repair, particularly in the repair of SSBs through the BER. The PARP family is composed of 17 enzymes that are involved in a variety of cellular processes including DDR, gene transcription, mitosis, and cell death. Among them, PARP-1 is the most abundant and well-defined protein involved in DNA repair. PARP-1 senses and binds SSBs, and creates poly(ADP-ribose) chains on itself and protein around the DNA damage. This creates a scaffold for other proteins that facilitate SSBs repair. The extensive auto-poly ADP ribosylation of PARP1 also results in its dissociation from the DNA, which is important for the completion of SSBs repair [63].

PARP inhibitors (PARPi) have shown encouraging results against HR defective-*BRCA1/BRCA2* mutant cancers, including ovarian and breast cancer. PARPi are thought to work by inducing accumulation of unrepaired SSBs, which eventually develop into DSBs after collision with DNA replication forks [64, 65]. PARPi may also inhibit dissociation of PARPs from SSBs, covalently trapping PARP to DNA damage sites [66]. Stabilized PARP-DNA complexes can block the DNA replication machinery and cause fork collapse and DSB formation. While normal cells with wild-type BRCA proteins utilize HR to repair these DSBs (Fig. 3), cancer cells with *BRCA1/BRCA2* mutations accumulate toxic DSBs and eventually undergo cell

death. Due to the selective toxicity of PARPi in *BRCA1/BRCA2* mutant cells, PARPi is an attractive agent for treating cancer associated with deleterious BRCA mutations.

Given that germline *BRCA* mutations have been linked to a higher risk of PDAC, PARPi (alone or in combination with other DNA damaging agent such as radiation or chemotherapy) could be an effective agent for treating *BRCA* mutated-pancreatic cancer. However, a study using a PDAC mouse model with a heterozygous *Brca2* mutation showed that loss of the wild-type *Brca2* allele is not necessary for PDAC development [47]. This suggests a possibility that not all PDAC in *BRCA2* mutation carriers loose the HR function. Consistent with this, *Brca2* heterozygote tumors in the mouse model were more resistant to a PARP inhibitor than *Brca2* null tumor cells [47]. These data, therefore, suggest that PARPi should be used to treat PDAC in *BRCA2* mutation carriers only after loss of the wild-type allele has been confirmed.

Because BRCA-associated pancreatic cancer is rare, there is limited data regarding the use of PARPi in pancreatic cancer. However, several investigations of PARPi in *BRCA1*/*BRCA2*-mutated pancreatic cancer patients have been instigated with encouraging partial response [67–69], and several clinical trials are ongoing.

Checkpoint Inhibitors

In response to DNA damage, normal cells undergo cell-cycle arrest at G1/S, intra-S, or G2/M. However, cancer cells often have inactive G1/S checkpoint due to *TP53* mutations (see Sect. 4.2.1). They are therefore highly dependent on the G2/M checkpoint for preventing mitotic entry with damaged DNA. As such, DNA damaging agents or replication inhibitors in combination with G2/M checkpoint inhibitors have been explored as therapeutic options for *TP53* mutant cancer. The G2/M checkpoint can be inactivated by inhibitors of the checkpoint kinase Chk1 or Wee1 (the kinase that phosphorylates and inactivates CDK1 in the G2/M checkpoint) (Fig. 1). The defective G2/M checkpoint will force cancer cells to enter mitosis with damaged DNA or incomplete DNA replication, causing mitotic catastrophe and cell death (see Sect. 4.2.3).

One study showed that the combination of chemotherapeutic agent gemcitabine and Wee1 inhibitor MK-1775 showed a synergistic antitumor effect in p53-deficient pancreatic cancer xenografts [70]. In another study, MK-1775 in combination with the PARP inhibitor olaparib and ionizing radiation showed an enhanced antitumor effect in pancreatic cancer [71]. However, it was recently shown that pancreatic cancer cell lines with deficiency in DNA repair genes (*BRCA2*, *FANCC,* and *FANCG*) were less sensitive to MK-1775 compared to DNA repair-proficient cells, suggesting that DNA repair proficiency of tumor cells is a critical factor for this therapy [72]. Similarly, the Chk1 inhibitor MK8776 was also shown to sensitize pancreatic cancer cells to a combination of gemcitabine and radiation, although the sensitization was specific to HR-proficient cells [73]. Taken together, although it requires intact DDR, chemotherapeutic agents in combination with inhibition of the

G2/M checkpoint can induce mitotic catastrophe and cell death in *TP53*-mutant pancreatic cancer.

Conclusion

The cell cycle is strictly controlled by multiple layers of regulation, yet findings from countless studies of cancer cells have uncovered its vulnerabilities. Many of the abnormalities in pancreatic cancer drive cell-cycle entry through elevated CDK4/6 activity. Loss of checkpoint functions and DNA repair defects are also common. The next challenge is to translate these findings to new therapeutic strategies for pancreatic cancer. Monotherapies promote drug resistance in cancer cells; therefore, combination therapies might be a more promising approach to this deadly disease.

Cross-References

- ▶ Apoptosis: Signaling Pathways in Pancreatic Cancer Pathogenesis
- ▶ Chemotherapy for Advanced Pancreatic Cancer
- ▶ EGFR (ErbB) Signaling Pathways in Pancreatic Cancer Pathogenesis
- ▶ Epigenetic Pharmacology
- ▶ Epigenetics and its Applications to the Progression Model of Pancreatic Cancer
- ▶ Familial Pancreatic Cancer
- ▶ Smad4-TGF-β Signaling Pathways in Pancreatic Cancer Pathogenesis
- ▶ The Molecular Pathology of Precursor Lesions of Pancreatic Cancer

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Pathologic Classification and Biological Behavior of Pancreatic Neoplasia

Olca Basturk, Michelle D. Reid, and N. Volkan Adsay

Contents

Abstract

Pancreatic neoplasms are classified according to the normal cells they recapitulate. These neoplasms' clinicopathologic and biologic characteristics are determined mostly by their cellular lineage. Most are of ductal lineage, characterized

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by tubular units, cysts, and papilla or mucin formation and expression of mucin-related glycoproteins and oncoproteins. There are also genetic and molecular alterations that are fairly tumor specific.

Invasive ductal adenocarcinoma (DA) constitutes the vast majority (>85%) of carcinomas of ductal lineage. DA is characterized by insidious infiltration and rapid dissemination, despite its relatively well-differentiated histologic appearance. Presumed precursors include microscopic intraductal proliferative changes now termed pancreatic intraepithelial neoplasia (PanIN). PanINs represent neoplastic transformation ranging from early mucinous change (low-grade PanIN) to frank carcinoma in situ (high-grade PanIN). A similar neoplastic spectrum characterizes intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs), cystic ductal-mucinous tumors with papillae formation, which may be associated with DA. As such, these are regarded as mass-forming preinvasive neoplasia. Some IPMNs are associated with colloid-type invasive carcinoma, a clinicopathologically distinct indolent tumor.

Although most ductal pancreatic neoplasia show some degree of mucin formation, serous tumors, of which serous cystadenoma is the sole example, lack mucin formation, presumably because they recapitulate centroacinar ducts.

Among non-ductal pancreatic tumors, neuroendocrine neoplasms are the most common. The vast majority are well-differentiated, low-/intermediate-grade malignancies characterized by protracted clinical course. In contrast, poorly differentiated neuroendocrine carcinomas (small or large cell type) are exceedingly uncommon and highly aggressive. Pancreatic acinar lineage tumors, namely, acinar cell carcinomas and pancreatoblastomas – the latter mostly a childhood malignancy – are uncommon and are associated with aggressive clinical course, though not as dismal as DA. Solid pseudopapillary neoplasm is a female-predominant pancreatic tumor of undetermined lineage that follows a predominantly indolent course.

Keywords

Ductal · Intraductal · Mucinous · Colloid · Acinar · Pancreatoblastoma · Solid pseudopapillary · Neuroendocrine

Introduction

Since the days of Galen of Ephesus, the “physician of physicians” (200 AD) had concluded that the pancreas was merely a fat pad serving as a protective cushion to the major vessels lying behind; the pancreas has remained an enigmatic organ, largely neglected by the medical field throughout the history. In the nineteenth century, it began to be appreciated as an organ, the failure of which leads to dire consequences. More importantly, it is now widely known that cancer arising from

the ductal system of this organ is one of the deadliest of all cancers and has recently become the fourth leading cause of cancer deaths in the USA [1]. This has led the medical field to analyze pancreatic neoplasia more carefully, and consequently, in the past two decades, various important developments have taken place in the pathologic classification, terminology, and our understanding of various pancreatic tumor types [2, 3].

Pathologic Classification of Pancreatic Neoplasia

Pancreatic neoplasms are classified according to which normal cell type of this organ they recapitulate, because the clinicopathologic and biologic characteristics of tumors are determined or manifested mostly by their cellular lineage.

The cell types that constitute the pancreas form three functionally distinct units:

1. Exocrine pancreas is responsible for the production and delivery of the digestive enzymes such as trypsin, chymotrypsin, amylase, and lipase to the duodenum. These enzymes are produced and stored in *acinar cells*. While acinar cells constitute the vast majority of pancreatic tissue (Fig. 1), neoplasms of acinar lineage are exceedingly uncommon. The second component of the exocrine pancreas is the ducts, the mere function of which is to transport the acinar enzymes to the duodenum. The ductal system begins with the *centroacinar cells* and continues with intralobar and interlobular ductules and, through the main pancreatic duct, ultimately opens into the ampulla of Vater. Although the ductal component is not a complex structure when compared with the other components, it is the main source of the vast majority of neoplasms in the pancreas [4]. This propensity for neoplastic transformation may not be very surprising as the ductal system is the only component in the pancreas that is exposed to the outside world (mutagens).
2. The second major and physiologically distinct component of this organ is the neuroendocrine, which is represented by widely scattered islands of neuroendocrine cells referred to as islets of Langerhans, distributed throughout the pancreas in forms of small, distinct nests amidst the acinar tissue (Fig. 1). The islets are responsible for producing a variety of hormones but mostly insulin, glucagon, and somatostatin, which play a key regulatory role not only in glucose metabolism but also other systemic metabolic processes as well. Unlike the exocrine component, which releases enzymes locally to the duodenum, the hormones produced by the neuroendocrine component are secreted to a rich capillary network that penetrates into the islets. Neuroendocrine tumors are not uncommon and form an important category, although they occur far less frequently than ductal neoplasia.
3. As in any other organs, there is also supportive tissue including fibroblasts, vessels, nerves, and immune cells in the pancreas, and these also, on occasion, give rise to pancreatic neoplasia [2–4].

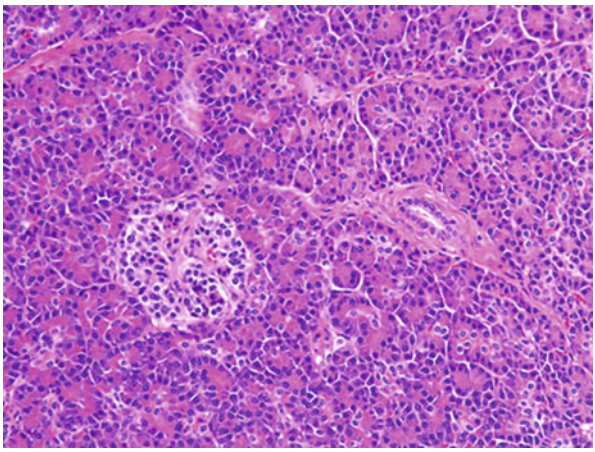

Fig. 1 Normal pancreatic tissue. Acinar cells arranged in lobules constitute the majority of the parenchyma. These cells have apical lightly eosinophilic cytoplasm due to the presence of zymogen granules and basophilia in the basal aspect of the cytoplasm. To aid in their secretory activity, the nuclei are polarized to the periphery and the cells are arranged in round units creating the *acinus*. In the *left middle part* of the field, an islet of Langerhans consisting of round collection of endocrine cells is represented. Endocrine cells have moderate amphophilic cytoplasm and nuclei with finely stippled chromatin pattern. In the *right upper part* of the field, an intralobular duct lined by cuboidal – low columnar – epithelium is seen

There are also tumors in the pancreas that are of undetermined origin and lineage. In the ensuing text, an overview of the clinicopathologic characteristics of pancreatic neoplasia will be discussed based on their lineage. Emphasis will be given to those that are more common ones.

Ductal Neoplasia

In order to transport the acinar enzymes to the duodenum, the ductal cells are organized in luminated structures and produce protective and lubricative glycoproteins (the mucins). Neoplasia of pancreatic ductal lineage recapitulates these characteristics at a variable degree. Tubular (lumen-forming) units, cysts (mega versions of these tubules), and papilla (fingerlike projections of the mucosa lining these ducts/cysts) are hallmarks of ductal differentiation in this organ and are also incorporated to the names of some of the tumors as well [2–4]. There are also certain genes, molecules, and mutations that are fairly specific to ductal neoplasia. Mucin-related glycoproteins and oncoproteins such as CA19-9, CEA, DUPAN, and MUC1 are typically detectable by immunohistochemistry in mucinous ductal tumors. Expression of certain subsets of cytokeratin such as CK19 and mutations in *KRAS* and *SMAD4/DPC4* genes are also fairly specific [5–7] and are typically lacking in acinar or neuroendocrine tumors with a few exceptions. Moreover, even though rare

scattered neuroendocrine cells can be seen in almost any ductal tumor; evidence of acinar differentiation such as enzyme activity is exceedingly uncommon.

Invasive Ductal Adenocarcinoma

More than 85% of pancreatic tumors are invasive ductal adenocarcinomas (DAs), also named as pancreatobiliary type, scirrhous, tubular, or usual ductal adenocarcinoma [4, 8]. Because it is by far the most common and most important tumor type in the pancreas, DA has become synonymous with "pancreatic cancer," which sometimes leads to erroneous interpretations due to inappropriate inclusion or exclusion of other cancers that occur in this organ but have different clinical, pathologic, and behavioral characteristics as discussed below. Patients with DA are usually between 60 and 80 years old (mean age: 63), and it is very uncommon to see this tumor in patients younger than 40 years old.

DAs grow rapidly, and regardless of the size of the tumor, metastasis to lymph nodes and liver ultimately ensue. They also have very insidious growth pattern, and in fact, along with ovarian cancer, DA is the most common cause of "intra-abdominal carcinomatosis," the formation of numerous small tumor nodules throughout the abdomen. It is also one of the most common sources of carcinomas of unknown primary. Only 20% of the cases with DA are resectable at the time of diagnosis.

Because of these features (rapid growth, insidious infiltration, and early dissemination), the cure rate of DA is extremely low, with a 5-year survival still 3–5% [1]. In fact, most 5-year survivors of "pancreatic cancer" prove to be a tumor type other than ordinary DA after careful reexamination of microscopic features [9].

The diagnosis of invasive DA can be very problematic, both at clinical and microscopic levels. This tumor type is typically associated with abundant host tissue stroma referred as *desmoplastic stroma* (Fig. 2). This creates a "scirrhous" (scar-like) appearance that can be very difficult to distinguish from true scarring inflammatory lesions of this organ, in particular, autoimmune and paraduodenal types of chronic pancreatitis. This difficulty in the differential diagnosis is also valid for microscopic examination. Injured native ducts of the pancreas can show substantial cytologic atypia that can closely imitate that of carcinoma, and conversely, most DAs form well-differentiated glandular units that resemble benign ducts [10, 11] (Fig. 3) or cause ductal obstruction and eventually lead to chronic pancreatitis. Consequently, the distinction of DA from pancreatitis is considered one of the most, if not the most, challenging differential diagnosis in diagnostic pathology.

However, DA has some morphologic characteristics that are fairly unique and not seen as much in other common organ cancers. First, despite its highly aggressive behavior, the vast majority of invasive DAs are "well or moderately differentiated" (Figs. 3 and 4), i.e., recapitulate the normal ducts extremely well. They also show a remarkable affinity to spread through the nerves and vessels. Nearly 80% of these cases show *perineural invasion* (Fig. 5) by microscopic examination, although if the entire tumor is examined, this ratio will probably be higher. This feature is thought to be the reason of back pain, one of the more common symptoms of this tumor. *Vascular invasion* is also very common and pancreatic carcinoma cells have this unique ability to form well-formed glandular elements in vascular spaces [12, 13]

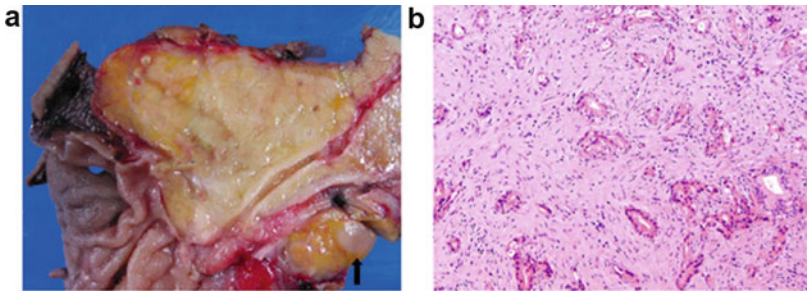

Fig. 2 (**a**) **Invasive ductal adenocarcinoma, macroscopic findings**. A firm, sclerotic, poorly defined mass is seen in the head of the pancreas. The rounded pale structure adjacent to the right lower border of the specimen represents a lymph node enlarged by metastatic adenocarcinoma. (**b**). **Invasive ductal adenocarcinoma** is characterized (and defined) by infiltrating tubular units embedded in **desmoplastic stroma**

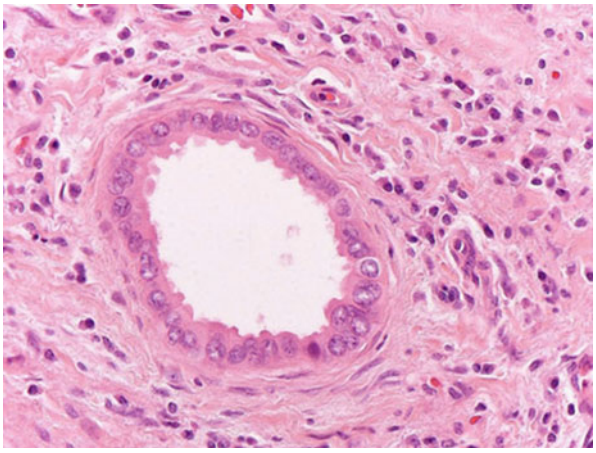

Fig. 3 Invasive ductal adenocarcinoma, well differentiated. Well-formed glandular structures lined by cuboidal cells closely mimic the nonneoplastic ducts

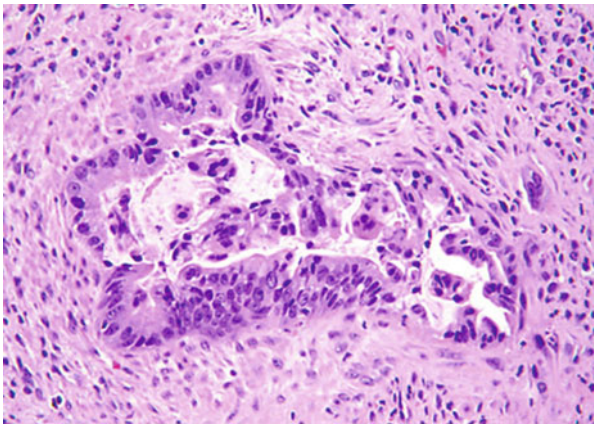

Fig. 4 Invasive ductal adenocarcinoma, moderately differentiated. There is a greater degree of cytologic and nuclear atypia. Loss of polarity can be seen as well

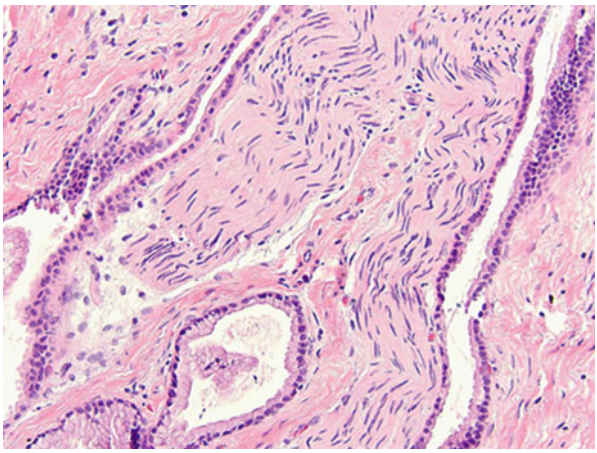

Fig. 5 Invasive ductal adenocarcinoma showing perineural invasion

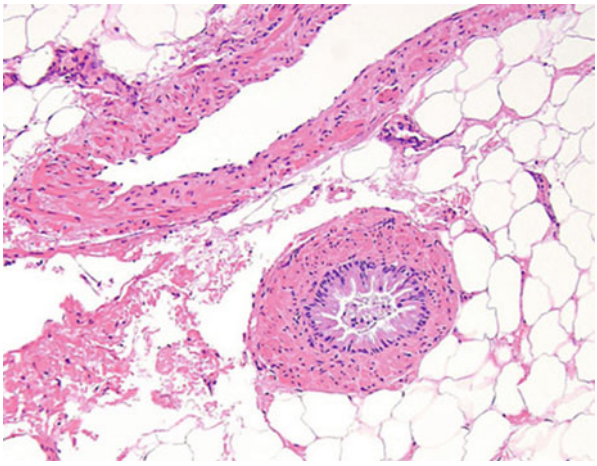

Fig. 6 Vascular invasion of infiltrating ductal adenocarcinoma. Carcinoma cells line the luminal surface of vascular walls in such an organized and polarized fashion that they form a well-structured duct-like unit virtually indistinguishable from normal ducts or PanINs

(Fig. 6). What referred to as *isolated solitary ducts*, which are microscopic (grossly invisible) invasive units located away from the main tumor lying individually in peripancreatic fat tissue, is a very common finding (Fig. 7) and may be responsible for the high recurrence rate of seemingly margin-negative resections [13].

The cells in DA are typically cuboidal shaped with variable amount of cytoplasm that contains mucin and mucin-related glycoproteins and may occasionally demonstrate predominance of a specific organelle creating distinctive patterns such as "foamy-gland" pattern with swollen, altered mucin, "clear-cell" pattern with abundant glycogen [14], and "oncocytoid" or "hepatoid" variants with prominent mitochondria or lysosomes, respectively [2, 3].

As discussed earlier, abundant *desmoplastic stroma* (Fig. 2) of variable cellularity is a very important feature of this tumor type. Carcinoma cells are somewhat diluted in this desmoplastic stroma, and this dilution phenomenon creates major problems

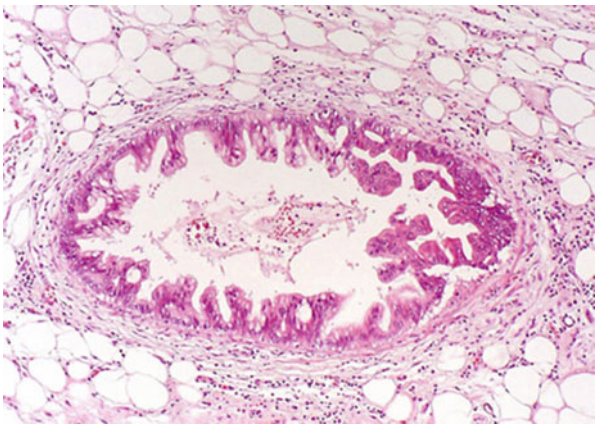

Fig. 7 Isolated solitary ducts surrounded entirely by adipocytes without any accompanying islets, acini, or other ducts are indicative of invasive carcinoma. This phenomenon of renegade ducts away from the main tumor is a peculiar manifestation of the insidious spread of pancreatic adenocarcinoma

for both diagnosticians and researchers. This is an important pitfall, in particular, for studies that utilize "global" arrays, which do not discriminate between the different cellular compartments of the specimen and analyze all pancreatic tissues together. If the intent is to analyze the carcinoma cells, it should be kept in mind that most of the tumor tissue is in fact composed of this desmoplastic stroma, not the cancer cells, and further complicating the analysis, the normal pancreas is also composed mostly of acini with no relevance to ductal carcinogenesis. Therefore, if a comparison of normal ducts and ductal adenocarcinoma is intended, normal ducts and carcinoma cells need to be dissected out from the background tissue, or alternatively, visual-aided methods of analysis such as immunohistochemistry or in situ hybridization are ought to be utilized by experts who can distinguish between the nonneoplastic and neoplastic elements.

Other Invasive Carcinomas of Ductal Lineage

Various uncommon types of invasive carcinomas of also ductal lineage, classified separately from the conventional DA, have been recognized [2, 3].

Colloid Carcinoma

Colloid carcinoma has been a well-established tumor type in other exocrine organs such as the breast where pure examples of this entity are associated with an excellent prognosis. In the pancreas, this tumor type has come to attention only after the delineation of IPMN (discussed below) as a distinct entity in the mid-1990s, because colloid carcinomas are seen in association with these tumors. It is characterized by extensive extracellular mucin deposition [15], which is responsible for its grossly soft, gelatinous appearance. By microscopic examination, there are mucin lakes that contain scanty clusters of carcinoma cells floating within this mucin (Fig. 8).

The prevailing theory is such that the mucin of colloid carcinoma is biochemically and biologically different than the mucins of other ductal cancers, made up of the "gel-forming mucin," the MUC2 glycoprotein [16]. It is speculated that, with its tumor suppressor properties and its physical distribution around the cells, this mucin

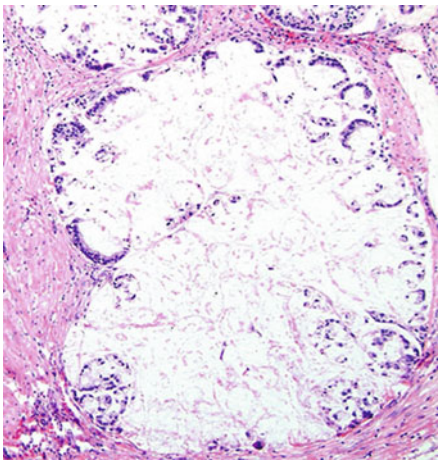

Fig. 8 **Colloid carcinoma** characterized by large amounts of mucin pools. Detached fragments of tumor cells can be observed in these pools

acts as a containing factor, limiting the growth and thus culminating in the more protracted clinical course observed in many studies [17].

Recent studies have also shown that molecular features of colloid carcinoma are different that those of DA. Colloid carcinoma, and its intestinal-type preinvasive precursor (intestinal-type IPMN), is associated with high frequencies of *GNAS* mutations [18].

Adenosquamous and Squamous Carcinoma

In the pancreas, squamous cells are found only rarely in injured ductal epithelium as a result of a metaplastic process. Same metaplastic phenomenon also seems to take place focally in some examples of DA. When this finding is prominent (arbitrarily defined as >25% of the tumor), the tumor is classified as adenosquamous carcinoma, and if the tumor is exclusively squamous, then squamous cell carcinoma. One may observe keratinization in various degrees in these tumors [19]. They constitute <2% of all invasive cancers of the pancreas and appear to be even more aggressive than ordinary DAs [20].

Medullary Carcinoma

This is an exceedingly uncommon tumor type as a primary in the pancreas [21], although it can occur in the periampullary region. The term medullary is adopted from similar tumors that occur in the GI tract. These tumors are often associated with a defect in DNA mismatch repair genes (genes that are responsible for correcting the mismatches that occur routinely in the DNA), which in turn leads to microsatellite instability [21]. Medullary carcinomas are characterized by nodular pattern and sheetlike growth of poorly differentiated epithelioid cells without any intervening stroma, as opposed to ordinary DAs, which have widely scattered well-formed tubular units with abundant stroma. In addition, there is often dense lymphoplasmacytic immune cell participation associated with medullary carcinomas.

Signet Ring Cell Carcinoma

Signet ring cell carcinoma is a tumor type that is well characterized in the stomach and is featured by a distinctive infiltration pattern referred to as "diffuse infiltrative." The carcinoma cells form small cords or chains of cells or invade as individual cells without any tubule formation. Commonly, this pattern is also associated with abundant intracytoplasmic mucin accumulation that pushes the nucleus to the periphery of the cell, which in turn creates the signet ringlike morphology [3].

Defined as such, signet ring cell carcinoma with all these characteristics is exceedingly uncommon in the pancreas. Many authors believe that those that are reported in the pancreas may very well represent secondary invasion from the stomach. Focal signet ringlike formations do occur in otherwise classical DAs of the pancreas; however, most authors feel that these should not be classified as signet ring carcinomas. Similarly, signet ring morphology may also be seen in colloid carcinomas of the pancreas, but in the absence of other characteristic features, these are not classified as signet ring carcinomas.

Undifferentiated Carcinoma

In some ductal carcinomas of the pancreas, the hallmarks of ductal differentiation, namely, the tubule formation, mucin production, and others, may be lacking. Such cases are classified as "undifferentiated carcinoma." Some can be so undifferentiated that only after adjunct studies such as immunohistochemical studies for keratins or mutation analysis for *KRAS* oncogene, the epithelial and ductal nature of the tumor can be elucidated. In some, epithelial-to-mesenchymal transition can be so complete that the tumor cells may be mostly spindle shaped and resemble sarcomas (i.e., sarcomatoid carcinoma). In fact, some may even show bone and cartilage formation. In others, the undifferentiated cells may form bizarre giant cells. These can be difficult to distinguish from high-grade malignancies like lymphomas or melanomas. Undifferentiated carcinomas are rare and their demographics do not seem to differ from ordinary DA [3].

Undifferentiated Carcinoma with Osteoclast-Like Giant Cells

Some sarcomatoid carcinomas of the pancreas have a peculiar predilection to attract osteoclastic-type giant cells of histiocytic/macrophagic origin [22], normally responsible for bone resorption. Often, osteoclastic cells are so abundant that they dominate the picture, and the tumor is referred to as "osteoclastic giant cell carcinoma." Recent molecular studies confirmed what is suspected by morphologic observations that these osteoclasts are in fact reactive in nature and that the malignant cells are actually the smaller, ovoid to spindle cells in the background (Fig. 9). Once inspected carefully, a more conventional adenocarcinoma component composed of invasive tubular elements is identified in most cases. Despite the impression in the literature, undifferentiated carcinoma with osteoclast-like giant cells appears to have a significantly better prognosis compared to DAs [22].

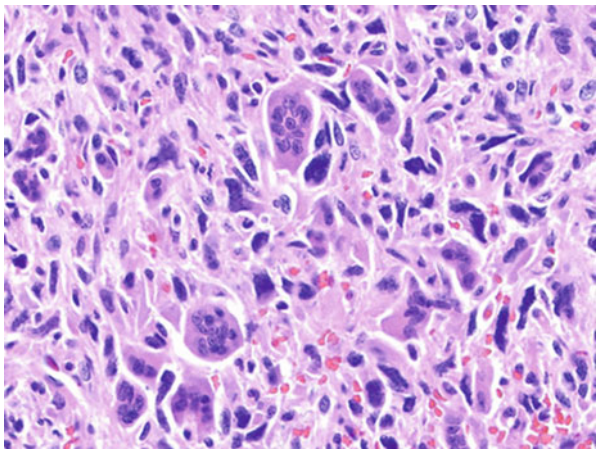

Fig. 9 Undifferentiated carcinoma with osteoclast-like giant cells. Nonneoplastic multinucleated giant cells (osteoclastic cells) of histiocytic origin are mixed with neoplastic mononuclear spindle-shaped/epithelioid cells. The mononuclear cells have hyperchromatic, occasionally bizarre nuclei

Noninvasive (Preinvasive) Ductal Neoplasia

Pancreatic Intraepithelial Neoplasia

It has long been recognized that there are abnormal *intraductal* proliferations that often accompany invasive DAs and may occasionally also be seen in the absence of DA. For decades, these were termed variably as hyperplasia, metaplasia, or dysplasia. In 1999, a group of pathologists interested in pancreatic neoplasia were brought together by the National Cancer Institute in a Think Tank that took place in Park City, Utah, and during that meeting, it was proposed to refer these lesions as *pancreatic intraepithelial neoplasia (PanIN)* [23]. Included in this neoplastic category as low-grade PanIN were changes that used to be called mucinous hypertrophy or mucinous metaplasia, based on the fact that although these mucinous changes seem perfectly innocuous and do not show classical morphologic attributes of neoplasia, they nevertheless exhibit some molecular alterations that are considered hallmarks of neoplastic change such mutation in *KRAS* oncogene. It has been shown that starting with these earliest forms of neoplastic transformation, the process advances to accumulate more genetic abnormalities including *p53* gene mutations [6, 24]. These genetic abnormalities are manifested microscopically as nuclear enlargement and hyperchromasia (deposition of abnormal nuclear material). Altered cellular metabolism leads to accumulation of different types of glycoproteins (mucins) as well as disorganization of cells, manifested as loss of polarity of the cells. Furthermore, loss of "guardians of genetic stability" leads to uncontrolled cellular proliferation that is reflected as increased mitotic activity. The spectrum of changes was previously graded as PanIN-1A, PanIN-1B, PanIN-2, and PanIN-3 [23]. However, recently, to improve concordance and to align with practical consequences, a two-tiered system (low grade vs. high grade) is proposed for all precursor lesions including PanINs, with the provision that PanIN-2 now be categorized as low grade [25]. High-grade PanIN is also regarded as synonymous to *carcinoma* in situ (Fig. 10), the last step before invasive cancer develops.

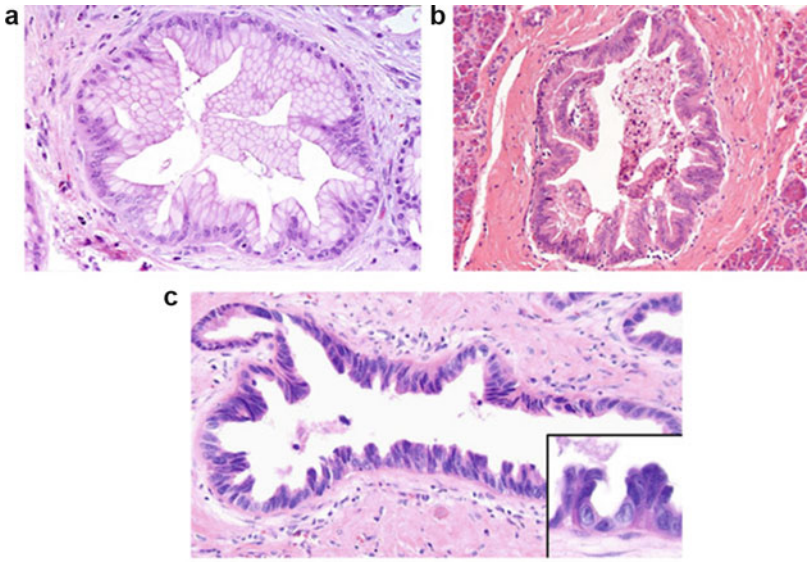

Fig. 10 (**a**, **b**) **Low-grade PanIN**, the normal cuboidal to low columnar ductal epithelial cells, is replaced by tall columnar cells containing abundant apical mucin. The nuclei are basally located. The epithelium can be relatively flat or papillary (**a**) in low-grade PanIN. Pseudostratification, loss of polarity, and mildly enlarged nuclei (**b**) may also be seen. (**c**) High-grade PanIN is characterized by severe cytologic atypia that is seen in full-blown carcinoma. Loss of polarity, nuclear irregularities, and prominent (macro) nucleoli (*inset*) and mitoses, which may occasionally be abnormal, are usually prominent

Mass-Forming Preinvasive Neoplasia

These lesions are in some ways similar to PanIN in the sense that they arise from the ductal system, and they are noninvasive neoplasia with potential for cancerous transformation. Unlike PanINs, however, they themselves present clinically with mass formation, usually as a cystic tumor [26–29], and this obviously raises the possibility of curative intervention. There are essentially three tumor types that can be included in this category of mass-forming preinvasive neoplasia: *intraductal papillary mucinous neoplasms* (*IPMNs*), *intraductal tubulopapillary neoplasms* (*ITPNs*), *and mucinous cystic neoplasms* (*MCNs*) [28].

These lesions are being encountered with increasing frequency and constitute up to 20% of pancreatic resections in some institutions [30], especially because they are often resectable tumors. The incidence of invasive carcinoma in these tumors is about 30%. Conversely, the estimated ratio for invasive pancreatic adenocarcinomas to arise in association with these lesions is about 1%. Even though there are controversies regarding their management, it is certain that these tumors are potentially curable, and because of this, the differential features of the lesions under this category and recognizing their clinicopathologic characteristics are important [28, 30].

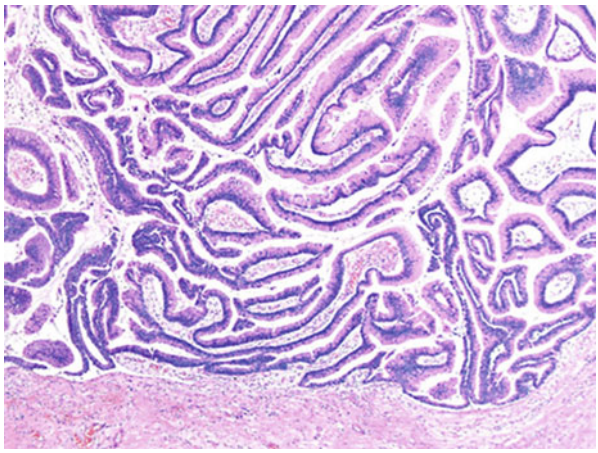

Fig. 11 Intraductal papillary mucinous neoplasia (IPMN). Tall, exuberant papillary structures lined by columnar cells with abundant mucin and cigar-shaped nuclei filling and dilating the ducts (cystic transformation). The overall picture of the process is highly similar to that of villous adenomas of the colon

Intraductal Papillary Mucinous Neoplasm

Intraductal papillary mucinous neoplasms (IPMNs) are characterized by intraductal proliferation of mucin-producing neoplastic cells that often form papillary configuration and lead to cystic dilatation of the ducts [25, 26, 29, 31–33] (Fig. 11). This process is reflected in imaging studies as dilatation of the ductal system with cyst formation and thus used to be called "ductectatic mucinous cystic neoplasm," and endoscopically, they are often associated with mucin extrusion from the ampulla of Vater, thus the previous name, "mucin-producing tumor."

IPMNs differ in the cell type that composes the papillary epithelium, allowing their stratification into intestinal, pancreatobiliary, gastric, and oncocytic subtypes [26, 34]. Although "oncocytic subtype" of IPMN was originally described as a separate variant of pancreatic intraductal neoplasms [35], the current (2010) WHO designated this neoplasm as a subtype of IPMN due to its overlapping features with other subtypes of IPMN [28].

There is also a spectrum of neoplastic transformation in IPMNs representing *adenoma-carcinoma sequence*, accompanied by an increasing number of molecular alterations [36]. Those with mild cytoarchitectural atypia are classified by the current (2010) WHO as low grade [28]. These are composed of relatively simple papillary units lined by well-polarized, tall columnar cells with basally oriented non-atypical nuclei and abundant apical cytoplasm with mucin. As the neoplasm progresses, with accumulation of other molecular-genetic alterations, the cells begin to show hyperchromatism and pleomorphism (variably sized and shaped nuclei), along with loss of organization and rapid proliferation of cells which lead to complex papillary elements, irregular clustering of cells, and cytologic atypia, altogether reflecting cancerous transformation, i.e., high-grade dysplasia or carcinoma in situ [23, 33]. The lesions in between the *low grade* and *high grade* were previously graded as *intermediate grade*. However, recently, a two-tiered system (low grade vs. high grade) is proposed for all precursor lesions to improve concordance and to align with practical consequences. In this two-tiered system, lesions with aforementioned

intermediate-grade dysplasia are categorized as low grade [25]. Accordingly, the term high grade is reserved only for the uppermost end of the spectrum.

The cancerous transformation within an IPMN culminates in invasive carcinoma in many patients. There are two types of invasive carcinomas that occur: (1) ductal (tubular) type [31, 32], which is virtually indistinguishable from conventional DA of the pancreas discussed previously and often behaves like one as well (with rapid recurrences, metastasis, and fatality) [37], and (2) colloid type [15], characterized by abundant extracellular mucin in which the carcinoma cells "float" (Fig. 8). Presumably, due to the containing effect of this stromal mucin, the spread of colloid carcinoma cells is much slower, and prognosis is significantly better than that of the ductal type [17].

Despite the earlier concerns and contentions, it has become clear in the past few years that if these tumors are carefully evaluated by experts and the possibility of high-grade dysplasia and invasive carcinoma is excluded definitively, this classification of IPMNs as low grade, high grade, or invasive is highly predictive of clinical outcome [37]. The important issue is that it is difficult to ascertain the absence of carcinoma without thorough pathologic examination of the tumors because foci of carcinoma can be focal and well hidden, not only from the eyes of the imagers on radiologic/endoscopic examination but even naked eyes inspecting the resected tumors in the pathology gross rooms, thus the mandate for complete microscopic examination of these lesions [29]. There are, however, surrogate findings that seem to be very helpful in preoperative classification of most (unfortunately not all) patients with IPMN. Most IPMNs confined to the branch ducts in the uncinate process tend to be small and less complex and prove to be low grade (i.e., without carcinoma) by pathologic examination [38]. The cell type of these *branch-duct-type* IPMNs also tends to be of gastric type [34]. Studies have shown that if a branch-duct IPMN is asymptomatic, smaller than 3 cm, and without mural nodularities (lack of complex papillary nodules) and EUS-guided cytologic examination fails to show any suspicious cells, the case can be managed conservatively because most of these prove to be low grade [30]. In contrast, branch-duct IPMNs that are larger and more complex with suspicious findings have a higher incidence of high-grade dysplasia and invasive carcinoma, which appears to justify surgery. IPMNs that also involve the main duct are referred to as *main duct type*. These have a high propensity to contain or evolve into invasive carcinoma and for this reason they typically warrant resection [30, 39]. Interestingly, these commonly show intestinal differentiation virtually indistinguishable from colonic villous adenomas [26, 34, 40]; in fact, some were previously reported as villous adenoma of Wirsung duct. This intestinal differentiation, which is also reflected at molecular level by expression of markers of intestinal programming, namely, MUC2 and CDX2 [34], as well as by recurrent mutations of *GNAS* [41, 42], is an intriguing and unique aspect of IPMNs. The problem is that these main duct IPMNs are also often diffuse, involving a large portion of or the entire pancreas; thus, their complete removal often means total pancreatectomy, which is an operation with relatively high complication rate, and it is difficult to balance the risk-benefit ratio in such patients, especially considering most IPMN patients

are relatively old (mean age: 68) with other comorbid conditions. Of note, many patients with IPMNs also have other neoplasms [32].

Intraductal Tubulopapillary Neoplasm

This is a recently recognized entity [43], first reported by Tajiri et al. under the heading of intraductal tubular carcinoma in 2004 [44, 45]. The entity is now being named *intraductal tubulopapillary neoplasm* [28], although papilla formation is seen only in a minority and in a very limited fashion in our experience.

The clinical findings are often indistinguishable from those of IPMNs. It occurs predominantly in the head but may involve any part of the gland [43, 46].

Microscopically, ITPNs are typically composed of multiple smooth-contoured nodules intervened with fibrotic stroma that may contain scattered pancreatic elements. The nodules are typically cellular but punctuated by numerous tubules, which are prominent in most cases. The overall pattern closely mimics intraductal variant of acinar cell carcinoma. Therefore, the absence of acinar markers is crucial to the diagnosis.

A third of the cases were reported to have invasive carcinoma of tubular type; however, distinguishing invasive carcinoma in ITPNs is a great challenge because of the complexity of the intraductal process and its striking ability to extend to atrophic lobules, creating a pseudoinvasive appearance [43]. Nevertheless, even if there is an associated invasive carcinoma, limited follow-up suggests these are indolent neoplasms with a protracted clinical course similar to IPMNs [43, 46].

Of note, genetic findings of ITPNs seem to be different than those of IPMNs. *KRAS* and *GNAS* mutations are very rarely, if ever, present in ITPNs, in contrast with IPMNs that frequently show these alterations [47–49].

Mucinous Cystic Neoplasm

Mucinous cystic neoplasms (MCNs) are seen almost exclusively in perimenopausal women (mean age: 48, >95% of the patients are female). They typically form a thick-walled multilocular cyst in the body or the tail of the pancreas [50–53]. Some examples may become infected and mimic pseudocysts. MCNs do not have obvious communication with the ductal system, which distinguishes MCNs from IPMNs. Cyst fluid is often rich in mucin-related glycoproteins and oncoproteins such as CEA, which may help differentiate these tumors from serous adenomas (see below) preoperatively. The cysts are lined by a mucinous epithelium, which may exhibit various degrees of cytologic and architectural atypias that have been classified as low, intermediate, and high grade [4]. However, recently, a two-tiered system (low grade vs. high grade) is proposed for all precursor lesions in the pancreas, including MCNs. In this two-tiered system, lesions with aforementioned intermediate-grade dysplasia are categorized as low grade [25]. Typically, small (<3 cm) and less complex lesions tend to be low grade, whereas larger, more complex lesions with abundant intracystic papillary nodules may harbor high-grade dysplasia or invasive carcinoma. As happened for IPMNs, it has become evident that if these tumors are examined thoroughly and the presence of high-grade dysplasia or invasive carcinoma is excluded, the grade does accurately predict the clinical outcome [50, 52] and

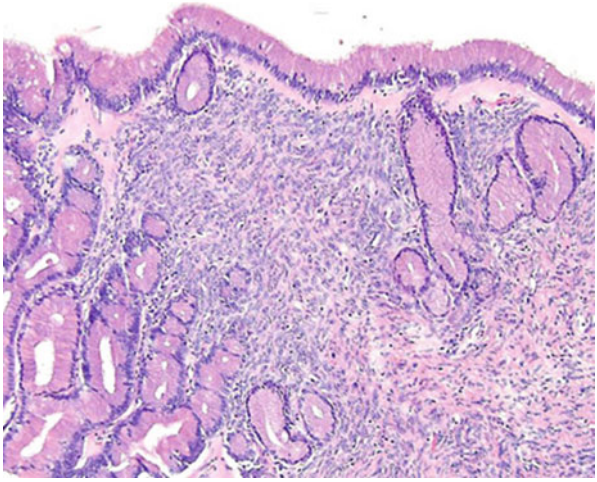

Fig. 12 Mucinous cystadenoma (MCN). The cyst lining is composed of tall columnar mucinous epithelium, surrounded by a cuff of distinctive hypercellular stroma on the wall which shows all the characteristics of ovarian stroma

that the cases classified as low-grade dysplasia are typically cured by complete removal. One caveat, however, is that invasive carcinoma may be very focal and easily missed if the tumor is not thoroughly examined, and for this reason, most authors advocate the total submission of these tumors for microscopic evaluation.

A microscopic feature that has become a requirement for the diagnosis of MCN is the presence of an "ovarian-type" stroma (Fig. 12) [25, 30, 39]. This stroma is not only similar to that of the ovarian cortex but also expresses estrogen and progesterone receptors that are detectable by immunohistochemistry, suggesting that hormones may have a role on initiation and progression of these tumors. This distinctive mesenchyme also helps distinguish MCN from other similar neoplasms, especially IPMNs.

Invasive carcinoma is seen in approximately 15% of the MCNs resected and is predominantly of the tubular type [53]. Interestingly, none of the cases have pure colloid-type invasion, which is the predominant type of invasion in IPMNs [53]. It appears that if there is established invasive carcinoma, the prognosis is often very poor [53].

Non-mucinous Ductal Neoplasia

Serous Cystadenoma

Serous cystadenomas (*SCA*), also called glycogen-rich or microcystic adenomas, are seen predominantly in older females (mean age: 58, F:M = 3:1) [54]. They appear to recapitulate centroacinar cells, and although they are of ductal lineage, they lack the features of mucinous differentiation. Grossly, they form well-demarcated, relatively large lesions (mean size: 4 cm; some up to 18 cm) with a central satellite scar [54]. *Microcystic* SCAs are typically composed of innumerable small cysts each measuring a few millimeters, which leads to the characteristic spongy appearance of the lesion by macroscopic examination, thus the name microcystic adenoma.

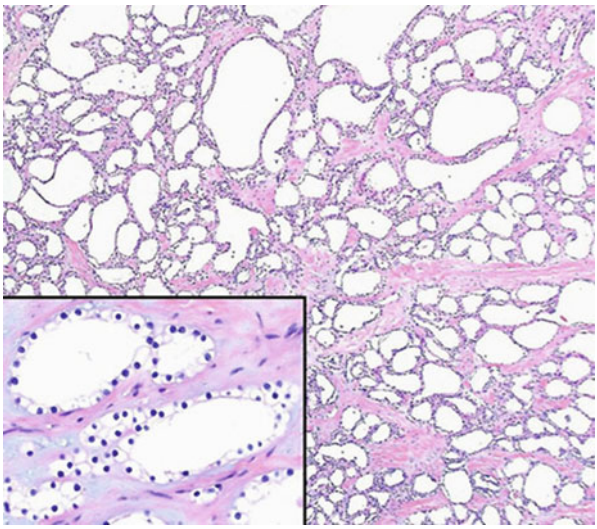

Fig. 13 Serous cystadenoma. Typical honeycomb (microcystic) pattern due to innumerable cysts of various sizes. Inset illustrates the lining of these cysts composed of low cuboidal epithelial cells with clear (glycogen-rich) cytoplasm showing distinctive, uniform, round, small nuclei with homogenous, dense chromatin

Macrocystic (unilocular or multilocular) and *solid* variants have also been described but are uncommon [54].

Microscopically, the microcysts correspond to variably sized gland-like structures lined by a single layer of non-mucinous cuboidal epithelium that contains intracytoplasmic glycogen that is responsible for the distinctive clear cytoplasm in the tumor cells (Fig. 13). The cysts contain watery, clear fluid that is devoid of mucin-related glycoproteins and oncoproteins in contrast with mucinous ductal tumors described above. This feature may be helpful in preoperative diagnosis. Multilocular macrocystic variant is characterized by a limited number of locules (typically <10), with each locule measuring in centimeters, creating a megacystic pattern, previously also called "oligocystic." Tumors with a singular locule are classified as unilocular macrocystic variant, and tumors with uniform, small, evenly shaped and sized nests or tubules with minimal or no lumen formation creating a solid, well-demarcated nodule on macroscopic examination constitute solid variant [54].

Of note, serous lesions may be observed in von Hippel-Lindau (VHL) disease [55] and some SCAs show *VHL* gene alterations [56]. Concurrent ductal adenocarcinomas, pancreatic neuroendocrine tumors, and congenital pathologic conditions may also be observed in association with SCAs [54].

Even though SCAs are invariably benign, it appears that a subset has a rapid doubling rate [57], which may be responsible for their large size in some patients. Also, larger serous neoplasms (>11.0 cm) with inflammation and hemorrhage may show localized adhesion and/or penetration of neighboring organs, including lymph

nodes, spleen, stomach, and colon, which does not seem to be an indicator of malignant behavior. There are only a few serous cystadenocarcinomas of dubious nature reported in the literature [58–60]. The majority of these are histologically identical to their benign counterparts. The only difference reported is that they recur, metastasize, or show angioinvasive growth. For those that are reported to recur or metastasize, the question of multifocality rather than true metastatic spread has been raised. Therefore, for serous neoplasms occurring in the liver, the possibility of synchronous-independent tumors may have to be considered before concluding metastasis.

Neuroendocrine Neoplasia

Aberrant neuroendocrine differentiation (the presence of scattered neuroendocrine cells or a small neuroendocrine component) is not uncommon in tumors with ductal differentiation (discussed above) and in acinar tumors (discussed later). However, if a tumor is predominantly composed of cells with neuroendocrine lineage, it is classified as "neuroendocrine."

Well-Differentiated Pancreatic Neuroendocrine Tumor

Well-differentiated pancreatic neuroendocrine tumors (PanNETs, previously referred to as "islet cell tumors" and "endocrine tumors") are the majority of the neuroendocrine neoplasms in the pancreas. They recapitulate the islets of Langerhans to variable degrees. Those that are associated with increased serum levels of hormones and lead to corresponding symptoms are referred to as "functional." These constitute nearly half of the PanNETs and are named according to which hormone they secrete (insulinoma, glucagonoma, gastrinoma, somatostatinoma, VIPoma, and others). Depending on the type and level of hormone secreted, the patients may suffer from a variety of symptoms or "syndromes." For example, insulinoma patients may present with symptoms related to excessive and erratic insulin secretion by the tumor that leads to "Whipple triad": (1) symptoms of hypoglycemia including confusion, convulsion, fatigue, and weakness, (2) serum fasting glucose level <50 mg/dL, and (3) relief of symptoms after intravenous glucose administration. Patients with "glucagonoma syndrome" have weight loss, diabetes mellitus, anemia, painful glossitis (sore and red tongue), venous thrombosis, and necrolytic migratory erythema. Excessive gastrin production may lead to Zollinger-Ellison syndrome characterized by multiple gastric and duodenal ulcers. Interestingly, the amount of hormone detected immunohistochemically in the tumor cells does not necessarily correlate with the functionality status [3].

In general, PanNETs form solid, circumscribed, fleshy lesions that appear significantly different than the scirrhous ductal adenocarcinomas. They can sometimes be multinodular. Microscopically, patterns suggesting a neuroendocrine differentiation are the result of a well-organized relationship of the neoplastic cells to numerous small blood vessels and the tendency of most cells to be rather uniform in appearance. The cells are usually rounded or polygonal, and in the

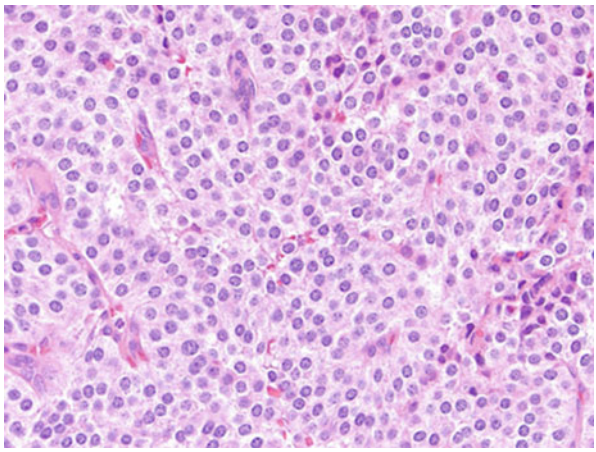

Fig. 14 Well-differentiated pancreatic neuroendocrine tumor. Uniform cells are arranged in nests, and nuclear features show the characteristic clumped, "salt and pepper" chromatin pattern

majority of cases, they are similar to one another in size and shape. The nuclei often resemble those of normal islet cells, often showing the distinctive "salt-and-pepper" chromatin pattern (with tiny clumps of dense heterochromatin scattered through the nuclei) (Fig. 14) [3].

In the current (2010) World Health Organization (WHO) classification system, pancreatic as well as gastrointestinal system neuroendocrine neoplasms are classified as *well-differentiated* neuroendocrine tumor (NET) (Grade 1 or Grade 2) and *poorly differentiated* neuroendocrine carcinoma (NEC) (Grade 3); see poorly differentiated neuroendocrine carcinomas section for the latter. This grading is performed on the basis of morphologic criteria and the assessment of proliferation fraction: [1] mitotic count and [2] Ki-67-labeling index (using the MIB1 antibody). Grade 1 NETs are defined as having a Ki-67 index of <3% and <2 mitoses/10 high-power fields (HPF). Grade 2 NETs have a Ki-67 index of 3–20% or 2–20 mitoses/10 HPFs [61]. For grade-discordant cases (based on differences in mitotic count and ki-67 index), the higher grade should be used [61].

Well-differentiated PanNETs (Grade 1 or 2) are low-grade malignancies. Those that are diagnosed at an early stage are often (but not always) curable, and even those that are advanced with metastases may have a relatively protracted clinical course that may stretch up to decades. Additionally, insulinomas also often follow a benign course since they are highly symptomatic even when they are small, thus detected in an early phase. PanNETs associated with multiple endocrine neoplasia, type 1 (MEN1), tend to be less aggressive as well. However, it should be kept in mind that a group of *well-differentiated* PanNETs have comparatively high Ki-67-labeling indices of more than 20% (usually between 20% and 50%) [62]. As per the current (2010) WHO guidelines, these *well-differentiated* PanNETs with an elevated proliferative rate are classified as NEC – Grade 3 – along with full-blown *poorly differentiated* NECs. However, preliminary studies have shown that even though their outcome is worse than that of ordinary *well-differentiated* PanNETs, it is still significantly better than that of the *poorly differentiated* NECs [62, 63]. Therefore, it

is becoming clear that the current Grade 3 group defined as >20% will have to be split into two separate categories in the future in order to distinguish the *well-differentiated* Grade 3 PanNETs (Ki-67, 20–50%) from the full-blown *poorly differentiated* NECs [62, 64].

Poorly Differentiated Neuroendocrine Carcinomas

As mentioned above, in the current (2010) WHO classification system, poorly differentiated pancreatic neuroendocrine carcinomas (NECs) are included in the Grade 3 category along with well-differentiated PanNETs that have more than 20 mitoses per 10 HPFs or a Ki-67 index greater than 20% [61]. This system suggests that poorly differentiated pancreatic NECs are part of a continuum with well-differentiated PanNETs, and therefore the two entities are closely related and that the grade should be based entirely on proliferation rate. However, evolving evidence strongly suggests that morphologic differentiation is also relevant and that poorly differentiated pancreatic NECs should be regarded as a separate entity [62, 63, 65].

Primary pancreatic poorly differentiated NECs are extremely rare, accounting less than 1% of all pancreatic [66] and at most 2–3% of all pancreatic neuroendocrine neoplasms [4]. Most patients are in their late 50s and there is a slight male predilection. In contrast to well-differentiated PanNETs, the poorly differentiated pancreatic NECs are not associated with hereditary syndromes and are usually clinically nonfunctional [65, 67].

Poorly differentiated pancreatic NECs are more common in the head of the pancreas and present as a large (median tumor size of 4 cm), relatively circumscribed, tan-yellow, fleshy mass. Microscopically, these carcinomas are subdivided into small and large cell variants, based on cell size. The small cell variant (small cell carcinoma) is characterized by small to intermediate cells with finely granular chromatin, high nucleus-to-cytoplasm ratio, inconspicuous nucleoli, prominent nuclear molding, and crush artifact [65]. The large cell variant (large cell NEC) is more common and characterized by large cells with prominent nucleoli and variable amounts of cytoplasm. Apoptotic cells and mitotic figures are abundant, but mitotic figures in the large cell NECs are usually not as numerous as in the small cell carcinomas [65]. In cases with the typical cytologic features of small cell carcinoma, it is not necessary to document neuroendocrine differentiation by immunohistochemistry. However, for large cell NECs, positive immunohistochemical staining for chromogranin or synaptophysin should be obtained to confirm the diagnosis [4, 61, 68].

Recently, pancreatic small cell carcinomas and large cell NECs were shown to be genetically related but distinct from well-differentiated PanNETs: The genetic changes frequently seen in these poorly differentiated pancreatic NECs, such as inactivation of the TP53 and the retinoblastoma/p16 pathways [69], are rarely observed in well-differentiated PanNETs [70]. Conversely, approximately 45% of sporadic well-differentiated PanNETs harbor mutually exclusive mutations in either *DAXX* (death domain-associated protein) or *ATRX* (α-thalassemia/mental retardation syndrome X-linked) genes [70]. *DAXX* and *ATRX* encode nuclear proteins, which form a chromatin-remolding complex and are involved in chromatin remolding at

telomeric and pericentromeric regions. Mutations of these genes are associated with loss of DAXX/ATRX protein expression.

The clinical course of poorly differentiated pancreatic NECs is worse than that of morphologically well-differentiated PanNETs that would be classified as (2010) WHO Grade 3 on the basis of proliferation rate [62]. Most cases are rapidly fatal with widespread metastases involving the regional and distant lymph node as well as intra- and extra-abdominal organs such as the liver and lung [63, 65]. Cisplatin- and etoposide-based regimens have shown some promise in controlling their growth; however, their overall prognosis remains grim [63, 71, 72] with a median survival of 11 months [65].

Acinar Neoplasia

Focal acinar differentiation can occasionally be observed as a small component of PanNETs and is also present as an important constituent of pancreatoblastomas (see below); however, most pancreatic tumors with predominant acinar differentiation are acinar cell carcinomas. With the exception of the recently described acinar cell cystadenoma, which is probably not a neoplasm (also called cystic acinar transformation [73], acinar differentiation is seen essentially only in malignant neoplasms in this organ.

Acinar Cell Carcinoma

Acinar cell carcinomas (ACCs) are rare neoplasms constituting <1% of all pancreatic carcinomas [74]. Occasionally, the neoplastic cells may secrete lipase and other digestive enzymes to the serum which may lead to the so-called lipase hypersecretion syndrome characterized by fat necrosis, polyarthropathy, occasional eosinophilia, and nonbacterial thrombotic endocarditis. Elevated levels of AFP in serum may also be observed.

Most ACCs are large (mean size: 8 cm) at diagnosis, and patients often present with early metastasis to the liver and lymph nodes. Macroscopically, they form a well-delineated, nodular, fleshy, yellow-tan tumor. Dense fibrotic appearance created by desmoplastic stroma characteristic of DAs is not a feature of ACCs [75]. Predominantly, intraductal growth of acinar cell carcinoma is uncommon but has been reported [76, 77]. Such cases can be mistaken as other intraductal neoplasias including intraductal papillary mucinous neoplasms or intraductal tubular carcinomas. On occasion, ACCs may present as cystic tumors.

Microscopically, ACCs are highly cellular tumors with solid sheets of cells that may form nests or rosette-like (acinar) patterns (Fig. 15). Many examples maintain production of digestive enzymes, which is represented as a distinctive eosinophilic granularity in the apical portions of their cytoplasms. These zymogenic granules are positive immunohistochemically by antibodies targeting specific enzymes such as trypsin, chymotrypsin, and BCL10 [74, 75, 78, 79]. Nuclei of ACCs are fairly round and relatively uniform. The most distinctive histologic feature of this tumor type is the presence of single prominent eosinophilic nucleolus (Fig. 15), recapitulating the

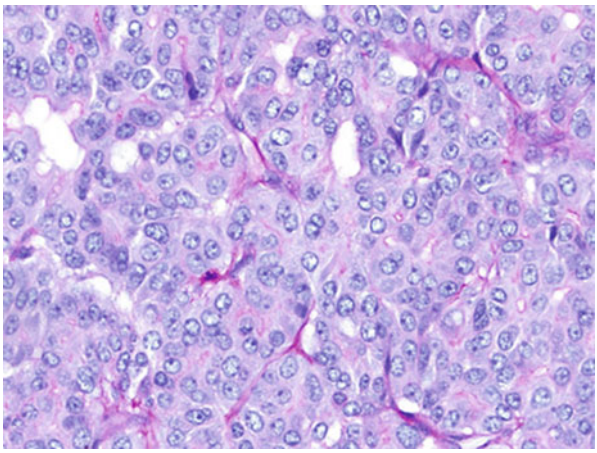

Fig. 15 Acinar cell carcinoma. The tumor cells are highly atypical but at the same time fairly monotonous and round. They display markedly chromopholic cytoplasm, mostly reflecting the enzymatic granules and cytoplasmic organelles involved in their production, which imparts this tumor its characteristic appearance. Single prominent nucleoli are also among the most distinctive histologic feature of this tumor type

normal acinar cells. Of note, aberrant and mixed differentiation, especially neuroendocrine, is quite common in acinar tumors (see below) [80].

In contrast to DAs, mutations in *KRAS*, *TP53*, and *SMAD4* are uncommon in ACCs [81]. However, molecular alterations in the APC-β-catenin pathway have been repeatedly reported in approximately 20% of ACCs, including inactivating mutations in *APC* and activating mutations in *CTNNB1* [82]. Recent whole exome sequencing and more targeted broad-spectrum sequencing studies have revealed a high degree of genomic instability in acinar neoplasms. Many different genes were mutated across the tumors studied, with no single gene being mutated in more than 30% of cases. The lack of common alterations in DA (*KRAS*, *SMAD4*, *TP53*, and *CDKN2A*), cystic neoplasm (*GNAS* and *RNF43*), and NET (*MEN1*, *DAXX*, and *ATRX*) genes was confirmed. Also confirmed were the alterations in *APC* and *CTNNB1* described previously. Additional recurrently altered genes include a variety of potential therapeutic targets, such as *JAK1*, *BRAF*, and genes of the mTOR and DNA repair pathways [83, 84]. An additional molecular alteration of potential therapeutic significance is the finding of *BRAF* fusions in 23% of acinar neoplasms [84]. The fusions are functional, leading to activation of the MAPK pathway, sensitive in vitro to MEK inhibitors. A rapid FISH assay to identify *BRAF* fusions in pancreatic acinar neoplasms has also been developed [85].

ACCs are fairly aggressive neoplasms. Liver metastases are seen in more than half of the cases and are mostly present at the time of diagnosis. Another 25% develop them subsequently [74]. Metastatic disease usually affects the lymph nodes and liver; even late in the course of disease, extrahepatic metastases are uncommon. However, rare cases present with ovarian metastases [86]. Nevertheless, the overall prognosis seems to be better than that of DA. Recent data have shown an even more favorable prognosis, presumably due to earlier detection and some responses to chemotherapy [87]. An overall 5-year survival rate of 43% (72% for patients undergoing resection, 22% for those with unresectable disease) and a median survival of 57 months for resectable disease and 20 months for those with metastases

are now reported [87]. However, most patients ultimately succumb to their disease. Prognostic factors include only staging features (primary tumor size, lymph node status, and presence of metastases) [75]. There is no predictive grading scheme for ACC.

Neoplasms with Multiple Lineages (Pancreatoblastoma and Mixed Acinar-Neuroendocrine Carcinoma)

Aberrant differentiation is exceedingly rare in the DAs, whereas it is rather common in non-ductal tumors.

Pancreatoblastoma is the principal example of the tumors with polyphenotypic differentiation; all three main constituents of normal pancreas, namely, acinar, ductal, and neuroendocrine, are represented in pancreatoblastomas, the acinar elements being the most consistent. In many ways, pancreatoblastoma can be regarded as pancreatic counterpart of other childhood "blastic" tumors such as Wilms (nephroblastoma), which is also a multi-lineage neoplasm. Pancreatoblastomas are very rare; however, they are the most frequent pancreatic tumor of the early childhood (mean age: 4). There appears to be a second peak in adults of 30's [88]. Elevated serum levels of AFP can be observed [89], and the tumors might be associated with Beckwith-Wiedemann [90] or familial adenomatous polyposis (FAP) syndromes [91].

Grossly these form large (7–18 cm), well-demarcated, solitary, solid, multilobulated tumors that can extend outside of the pancreas. Microscopically, solid sheets, nests, trabecula, and strands of neoplastic cells are divided by variable amounts of stroma, which on occasion may contain heterologous elements such as osteoid. Necrosis may be present. Squamoid corpuscles composed of large, spindled squamoid cells that form small morular arrangements, occasionally with keratinization, are a pathognomonic finding of pancreatoblastoma (Fig. 16), not seen in other tumor types of the pancreas [88, 92]. Acinar, ductal, and neuroendocrine elements can be highlighted by the markers discussed in the corresponding sections. The squamoid nests do not seem to reflect a particular cell lineage. Interestingly, they also show nuclear labeling for β-catenin, which is implicated in the molecular histogenesis of acinar neoplasms, but the acinar components of the tumors retain normal membranous labeling for β-catenin [93]. Genetic alterations are similar to those of ACCs and starkly different from the ones seen in DAs [78].

Pancreatoblastomas can behave aggressively, but like pediatric ACCs, they are less aggressive in children than in adults. Patients without metastases at presentation may be cured, and favorable responses to chemotherapy have been documented [79, 88, 94].

In addition to pancreatoblastoma, prominent multi-lineage differentiation is also seen in tumors that are classified as "mixed" [95]. While ACCs often show focal aberrant neuroendocrine differentiation in forms of microfoci or scattered individual cells, in some cases, there is a well-established, prominent neuroendocrine component. If this component constitutes more than 25% of the tumor, the designation of

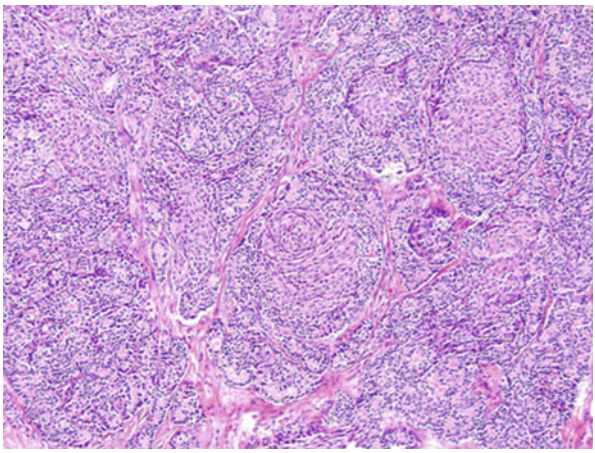

Fig. 16 Pancreatoblastoma. The acinar component predominates in most pancreatoblastomas as seen here. The most distinctive and characteristic finding in this tumor type are the *squamoid corpuscles*, which are well-defined nests of plump to spindle-shaped cells that form a vague fascicular or whorled pattern highly similar to the "morules" seen in other malignant tumors that are also related to beta-catenin pathway alterations

"mixed acinar- neuroendocrine" carcinoma is given [4]. Similarly, on occasion, ACCs may have a significant ductal component, and if this component is >25% of the tumor, the diagnosis of "mixed acinar-ductal" carcinoma is rendered. It may be important to note that in these "mixed" carcinomas, invariably the dominant component is acinar. Mixed carcinomas are very rare; thus, their clinical behavior is difficult to ascertain, but most appear to behave like ACCs (discussed above) [4].

Neoplasms of Uncertain Histogenesis

Solid Pseudopapillary Neoplasm

Solid pseudopapillary neoplasm (SPN) is a peculiar tumor of indeterminate lineage. This is reflected in the various descriptive names previously used for this tumor: "papillary cystic," "cystic and papillary," "solid and cystic," and "solid and papillary" [96]. It is also known as Frantz or Hamoudi tumor, crediting the observers that recognized this as a distinct category.

Clinically, SPNs are significantly more common in women. In a recent series, 84% of the patients have been reported to be females [97]. They have been described in all age groups, but the mean age is 33 [97, 98]. Symptoms are nonspecific, and some cases are detected incidentally following trauma or during gynecologic or obstetrical exams. As experience with these relatively uncommon tumors developed, it became clear that these are essentially solid tumors, which often undergo cystic degeneration [98, 99]. Unlike in other cystic tumors, the cysts are not lined by an epithelium. Grossly, their appearances vary from beige-tan to brown-hemorrhagic

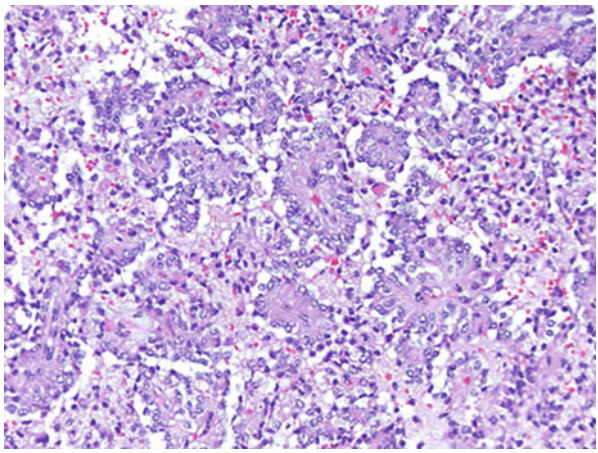

Fig. 17 Solid pseudopapillary neoplasm. Prominent pseudopapillary growth pattern is seen in most cases and is a characteristic feature of this enigmatic tumor

depending on the degree of hemorrhage and degeneration. Histomorphologically, SPNs typically show diffuse cellular proliferation of relatively bland-appearing cells admixed with variable degree of stroma ranging from dense collagen to myxoid to hemorrhagic (Fig. 17). The cells can also be arranged in vague nests, intervened by fairly dense but relatively inconspicuous microvasculature. The preferential dyscohesiveness of the cells away from the microvasculature, presumably related to the alterations in cell adhesion molecules (catenins and cadherins) [99, 100], leads to the highly distinctive arrangement of cells that is referred to as "pseudopapillary," which was recently incorporated to the name of this entity, although it is not present in all cases. Other characteristic and rather specific findings include nuclear grooves and the eosinophilic cytoplasmic globules.

Despite intensive study, the line of differentiation of these neoplasms remains uncertain [98]. Although some cases appear to exhibit some less specific neuroendocrine differentiation markers such as synaptophysin or CD56, chromogranin is never expressed. Both acinar and ductal markers are also consistently negative. In fact, the weak expression of keratin casts doubt on even the epithelial nature of these tumors, although some authors classify them as "carcinoma." There are various markers expressed by this neoplasm that were thought could be helpful in its diagnosis and also in establishing its lineage; however, none are specific. These include vimentin, alpha-1-antitrypsin, progesterone receptors, beta-catenin, and CD10. Among these, beta-catenin expression appears to be most helpful because it is not seen in neuroendocrine tumors, which is the main differential. This pattern of labeling is secondary to constitutive activation of Wnt pathway in SPN that caused point mutations within exon 3 of the *CTNNB1* gene (reported in more than 90% of SPNs) [42]. Recent molecular studies have also shown the absence of abnormalities in *KRAS*, *TP53*, or *SMAD4* genes observed in DA and support also that SPN is distinct from all other pancreatic neoplasms [6]. However, it should be noticed that genetic/mutations in the beta-catenin/APC pathway are seen in up to 80% and 50% of pancreatoblastomas and ACCs, respectively [42, 82].

SPNs are considered malignant, but metastases occur in only 10–15% of cases [98, 101]. In almost every instance, metastases are either in the liver or peritoneum; nodal metastases are rare. Interestingly, even patients with metastatic disease often survive for many years (even decades) with few symptoms [97, 102, 103]. In fact, only rare deaths have been attributed to direct effects of SPNs [97]. None of the pathologic findings, with the exception of anaplastic/sarcomatoid differentiation, have been proven of value in determining which rare cases will have metastasis [101].

Miscellaneous Cystic Pancreatic Lesions

In contrast to the ones seen in salivary glands, *lymphoepithelial cysts* (LEC) of the pancreas [104] do not show any association with immune-suppressive, autoimmune, or malignant diseases. They are mainly seen in adult men (mean age: 52, M/F = 3/1) [104, 105] and usually are asymptomatic and incidental lesions, which can be located within the pancreas or protrude from the pancreas and present as a peripancreatic mass. Gross examination reveals a well-demarcated, often encapsulated, uni-/multilocular cystic lesion with semisolid, caseous, keratinaceous, or sometimes watery luminal contents. Histologically, the cysts lined by mature stratified squamous epithelium with variable keratinization are surrounded by a band of dense lymphoid tissue, which may show lymphoid follicle formation. Lymphoepithelial islands can also be seen in some cases. Leakage of the cyst content might cause inflammatory reaction and granuloma formation in the surrounding tissue. Cholesterol clefts and fat necrosis can be seen as well. LEC-like epidermoid cysts may evolve in intrapancreatic accessory spleens [2, 3].

Other entities that may form cystic lesions are the following: *Dermoid cysts* [104, 105] are similar to LECs but lack lymphoid tissue and have skin adnexal elements including sebaceous glands. *Lymphangiomas* [106] are seen in young females (mean age: 29, M/F = 1/3) and form endothelial-lined cysts surrounded by a rim of lymphoid tissue. *Squamoid cyst of pancreatic ducts* is a recently described entity that is probably reactive in nature but may produce high CEA levels [107]. *Congenital cysts* and *intestinal duplications* may also form cystic lesions in the vicinity of the pancreas and periampullary region. These may have variable lining including respiratory type, intestinal, squamous, or transitional [3].

Mesenchymal Tumors

Mesenchymal tumors including fibromatosis (desmoid tumor), solitary fibrous tumor, leiomyoma, schwannoma, primary sarcomas such as primitive neuroectodermal tumor, synovial sarcoma, desmoplastic small round cell tumor, leiomyosarcoma, malignant fibrous histiocytoma, and others may rarely arise primarily in the pancreas [108].

Pseudotumors

In the pancreas, a variety of nonneoplastic conditions may form solid masses that may mimic cancer. Up to 5% of pancreatectomies performed with the preoperative clinical diagnosis of carcinoma prove to be nonneoplastic by pathologic examination, although this figure has been on a steep decline in the past few years with improved preoperative diagnostic modalities and the experience in their usage [109]. Chronic inflammatory lesions are the leading cause of pseudotumor formation, and among these, two entities remain highly problematic as close mimickers of cancer [2–4]:

1. Autoimmune pancreatitis (AIP), a relatively recently defined distinct form of pancreatitis, has been divided into two types – type 1 and type 2 – which share certain clinical similarities but are vastly different in terms of pathology and extrapancreatic features [3].
   (a) Type 1 (previously known as *lymphoplasmacytic sclerosing*) autoimmune pancreatitis is characterized by a pseudotumor composed of dense lymphoplasmacytic infiltrates, in particular IgG4-positive plasma cells, which concentrate around the ducts ("duct-centric pancreatitis") as well as medium-sized venules (periphlebitis) and is associated with fibrosis. The process may be associated with diffuse enlargement of pancreatic tissue or may form a localized lesion. The pancreas was the first organ in which IgG4-related disease was identified, but the disease has now been described in virtually every organ system: the biliary tree, meninges, orbital tissues, salivary glands, thyroid gland, lungs, etc. [3]. The serum IgG4 concentration is elevated (>135 mg/dL) in many patients, which can be very helpful in distinguishing it from carcinoma [109–111], but it may be normal in up to 40% of patients with biopsy-proven AIP type 1 [112]. On immunohistochemistry, the majority of plasma cells are positive for IgG4. The finding of more than 50 IgG4 (+) plasma cells/HPF is considered highly specific for AIP type 1. To identify the full spectrum of changes occurring in AIP, one must recognize its five cardinal features (the Mayo Clinic's HISORt criteria): suggestive **h**istology showing lymphoplasmacytic infiltrate with storiform fibrosis, **i**maging showing a diffusely enlarged pancreas, **s**erology showing elevated IgG4 levels, or evidence of **o**ther organ involvement and **r**esponse to steroid therapy [113, 114].
   (b) AIP type 2 seems to be a pancreas-specific disorder. It is not associated with either other organ involvement or with serum IgG4 elevation typically seen in AIP type 1. However, the lack of other organ involvement or absence of serologic abnormalities in patients with AIP does not necessarily imply the diagnosis of type 2, as type 1 also can be without other organ involvement and seronegative. The most distinctive feature of the AIP type 2 is a dense periductal lymphoplasmacytic inflammation accompanied by neutrophilic microabscesses within the lumen, the so-called granulocytic epithelial lesion

(GEL), involving medium-sized and small ducts, as well as in acini [110, 115–118]. AIP type 2 cases have none or very few (<10 cells/HPF) IgG4 (+) plasma cells [115].

Regardless of subtype, it is important to recognize AIP because it is considered a reversible pancreatitis. The pancreatic (and extrapancreatic) manifestations respond to steroid therapy within an interval of a few months [119]. Although relapses are common, especially in AIP type 1 [120], retreatment with steroids remained effective at inducing remission [116, 119].

2. Paraduodenal pancreatitis, also referred to as cystic dystrophy of heterotopic pancreas, paraduodenal wall cyst, or groove pancreatitis, typically forms thickening, nodularities, and stricture of duodenal wall at the region of accessory ampulla and resembles periampullary cancers [121]. The lesion is characterized by dense myoid proliferation of stroma admixed with pancreatic ducts, rounded acinar lobules, extravasated acinar secretions that illicit stromal and inflammatory reaction rich in eosinophils, as well as Brunner's gland hyperplasia. Most patients are middle aged and have history of alcohol abuse. It is hypothesized that paraduodenal pancreatitis forms as a result of localized alcoholic pancreatitis differentially involving the region drained by the accessory duct [11, 109].

Other lesions that may form pseudotumor and mimic cancer are the following [109]: *Adenomyomatous hyperplasia of ampulla of Vater* is a subtle lesion that is difficult to define; larger examples (>5 mm) have been found to be the cause of obstructive jaundice. *Accessory (heterotopic) spleen* may also form a well-defined nodule within the tail of the pancreas and is typically mistaken for neuroendocrine neoplasm.

Lipomatous hypertrophy is the replacement of pancreatic tissue with mature adipose tissue that occasionally leads to moderate to marked enlargement of the pancreas [122]. *Hamartomas* are very rare if the entity is defined strictly. They are characterized by irregularly arranged mature pancreatic elements admixed with stromal tissue. A cellular, spindle-cell variant with c-kit (CD117) expression is recognized.

*Pseudolymphoma*s form well-defined nodules composed of hyperplastic lymphoid tissue. Rarely, foreign body deposits, granulomatous inflammations (such as sarcoidosis or tuberculosis), and congenital lesions may form tumoral lesions [4].

Secondary Tumors

Secondary tumors involving the pancreas can be listed according to the decreased frequency as pulmonary tumors, lymphomas, gastrointestinal tract carcinomas, renal cell carcinomas, and breast carcinomas [123]. The majority are detected only at

autopsy [124]. Tumors arising in the retroperitoneum, nearby lymph nodes, or gastrointestinal system may also show direct extension to the pancreas. Lymphomas and renal cell carcinomas involving the pancreas are more prone to mimic primary cancers [123]. Renal cell carcinomas may even form polypoid ampullary lesions and may grow within ducts.

Conclusion

The vast majority of pancreatic neoplasms are of ductal lineage rather than neuroendocrine or acinar; thus most research focuses on the ductal tumors. Consequently, significant developments have taken place in the classification and in our understanding of ductal neoplasia in the recent years.

A major recent development was the more unified terminology and grading of precursor lesions, namely, pancreatic intraepithelial neoplasia (PanIN) comprising a neoplastic transformation ranging from early mucinous change (low-grade PanIN) to frank carcinoma in situ (high-grade PanIN).

Also, it is now well known that even the different types of ductal neoplasia vary greatly in their clinicopathologic characteristics and prognoses. Although invasive ductal adenocarcinoma, the most common carcinoma occurring in the pancreas, is one of the deadliest of all cancers, cystic lesions are often either benign or low-grade indolent neoplasia. Better characterization of cystic ductal tumors such as intraductal papillary mucinous neoplasms and mucinous cystic neoplasms has been a major step not only from the standpoint of patient care but also for cancer researchers, because they serve as an interesting model of carcinogenesis. They have well-established malignant potential, representing an *adenoma-carcinoma sequence* that often culminates in invasive carcinoma. Invasive carcinomas in intraductal papillary mucinous neoplasms are predominantly colloid type, which is now regarded as clinicopathologically distinct type of pancreatic cancer with indolent behavior.

Among non-ductal tumors of the pancreas, neuroendocrine neoplasms are by far the most common and form an important category. The majority of these are low-intermediate grade malignancies, and their behavior is far better than that of invasive ductal adenocarcinoma. Those that are treated at an early stage are even considered "benign." However, it should be noted that poorly differentiated neuroendocrine carcinomas are highly aggressive and rapidly fatal tumors.

Key Points

- Pancreatic neoplasms are classified according to which normal cell type of this organ they recapitulate (ductal, acinar, endocrine), because the clinicopathologic and biologic characteristics of tumors are determined or manifested mostly by their cellular lineage.

- Most pancreatic neoplasms are of ductal lineage. Invasive ductal adenocarcinoma (DA) constitutes the vast majority (>85%) of carcinomas of ductal lineage. These are rapidly progressive and highly aggressive solid tumors despite their relatively well-differentiated appearance. They have a tendency to illicit abundant desmoplastic stroma and high propensity for perineural invasion and vascular spread.
- In contrast with solid tumors, cystic lesions of the pancreas are often either benign or low-grade indolent neoplasia. However, those that are mucinous, namely, intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs), constitute an important category, because they have well-established malignant potential, representing an *adenoma-carcinoma sequence*. Approximately 30% of resected IPMNs and 20% of all MCNs have an associated invasive carcinoma. Invasive carcinoma in IPMNs is predominantly colloid type, and those associated with MCNs are almost exclusively of the ordinary ductal type.
- Among non-ductal tumors, well-differentiated pancreatic neuroendocrine tumors (PanNETs) are by far the most common. These are much more indolent tumors than DA and can be associated with multiple endocrine neoplasia, type 1 (MEN1). They form solid, circumscribed, fleshy lesions. Microscopically the tumor cells mimic the islet cells.

Future Scientific Directions

- Most of the DA tissue is composed of desmoplastic stroma and not the cancer cells. Therefore, if the intent is to analyze the carcinoma cells, carcinoma cells need to be dissected out from the background tissue, or alternatively, visual-aided methods of analysis such as immunohistochemistry or in situ hybridization are ought to be utilized.
- *Preinvasive* neoplasms (PanINs, IPMNs, ITPNs, and MCNs) constitute a very important category not only because they are early cancers and thus catching in an early stage often leads to cure, but also they offer an invaluable model of carcinogenesis to analyze. They all show a spectrum of cytoarchitectural atypia. It is now known that starting with the earliest forms of neoplastic transformation, the process advances to accumulate genetic abnormalities. Some of these abnormalities are well documented in the literature, but a lot more awaits to be elucidated.
- On the cyst wall and septa of MCNs, a distinctive ovarian-type stroma that regularly expresses progesterone receptors and sometimes estrogen receptors is seen. This stroma is an entity-defining feature of these neoplasms, to an extent that it has become a requirement for the diagnosis. Moreover, some MCNs are reported to be associated with ovarian thecomas. Efforts should be made to further elucidate the nature of this stroma and hormone influence in the pathogenesis of these neoplasms.

- Despite intensive study, the line of differentiation of solid pseudopapillary neoplasm remains uncertain.
- Currently, it is difficult to determine which well-differentiated pancreatic neuroendocrine tumors will have recurrences and metastases. More studies are needed to more accurately estimate the malignant potential of a given well-differentiated pancreatic neuroendocrine tumors.

Clinical Implications

- Invasive ductal adenocarcinoma cases have highly insidious infiltrative patterns, and often the carcinoma cells are spread far beyond the seemingly confines of the main tumor. Perineural invasion is common and is thought to be the reason of back pain, one of the more common symptoms of this tumor.
- It has become clear in the past few years that if IPMNs are carefully evaluated and the possibility of high-grade dysplasia (*carcinoma* in situ*)* and associated invasive carcinoma is excluded definitively, the classification of IPMNs as low-grade, high-grade, or with an associated invasive carcinoma is highly predictive of clinical outcome. This is also valid for MCNs.
- There are surrogate findings that seem to be very helpful in preoperative classification of most patients with IPMN. Most IPMNs confined to the *branch ducts* in the uncinate process tend to be small and less complex and prove to be adenomas by pathologic examination. In contrast, IPMNs involving main ducts are usually larger and more complex with suspicious findings and have a higher incidence of malignancy.
- Well-differentiated pancreatic neuroendocrine tumors are low- or intermediate-grade malignancies. Those that are diagnosed at an early stage are often curable. Even those that are advanced with metastases may have a relatively protracted clinical course. It should be noted here though that poorly differentiated neuroendocrine carcinomas are highly aggressive and rapidly fatal tumors.
- Nearly half of the well-differentiated pancreatic neuroendocrine tumors are associated with increased serum levels of hormones and are named according to which hormone they secrete (insulinoma, glucagonoma, gastrinoma, somatostatinoma, VIPoma, etc.). Depending on the type and level of hormone secreted, the patients may suffer from a variety of symptoms or "syndromes."

Cross-References

► Controversies in Pathology Reporting and Staging
► Molecular Pathology of Pancreatic Endocrine Tumors
► The Molecular Pathology of Precursor Lesions of Pancreatic Cancer

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Developmental Molecular Biology of the Pancreas

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Contents

Abstract

Pancreatic organogenesis is a complex and coordinated process that generates a compound gland of exocrine tissue composed of acini and ducts and endocrine tissue organized in islets of Langerhans. Both tissues originate from the same early endodermal epithelium through cell-cell signaling exchanges with adjacent tissues, including associated mesenchyme that directs a cascade of transcriptional

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regulatory events. Current research is aimed at elucidating the formation of pancreatic cell types and the molecular mechanisms that shape the anatomy and physiology of the pancreas. Insights into these questions come from a combination of mouse and human genetics and, increasingly, pluripotent stem cell-based models of organogenesis. These studies have identified both intrinsic factors, such as transcriptional regulators, and extrinsic signaling factors, such as secreted growth factors, morphogens, and cell-surface ligands, as determinants of cellular fate decisions, proliferation, or differentiation. The interplay between organ-restricted intrinsic factors and widely used extrinsic factors guides the stepwise process of pancreatic development from early endodermal patterning and specification of the initial pancreatic field to expansion of pools of progenitors, resolution of individual cell types, and the differentiation of mature exocrine and endocrine cells. A better understanding of pancreatic development is proving useful for comprehending the regulatory defects that drive pancreatic carcinogenesis and for devising effective therapies to correct those defects.

Keywords
Pancreatic development · Acinar development · Ductal development · Cell delamination · Epithelial plexus · Pancreatic fate · Multipotent progenitors · Epithelial to mesenchymal transition

Overview of Pancreatic Development

The mammalian pancreas is a compound gland of exocrine and endocrine epithelia. In adults, the exocrine compartment is composed of ducts and acini and comprises ~90% of the mass of the gland. The endocrine compartment is organized as islets of Langerhans and comprises ~2% [2]. These two tissues serve two distinct functions: (1) the production of digestive enzymes, which are secreted from the acinar cells and channeled to the duodenum via the ducts and (2) the regulation of blood sugar levels by the endocrine cells of the islets of Langerhans via the islet vasculature. A description of the embryonic formation of the pancreas must include the genesis of both exocrine and endocrine tissues, as well as the mechanisms that distinguish these two developmental programs and balance the proportion of precursor cells committed to each. The organogenesis of the pancreas has been well characterized for mouse, rat, rabbit, and chicken. Although pancreas development has been less well studied in the human embryo in situ, it is increasingly amenable to modeling in vitro with human pluripotent stem cells (PSCs). The genetic toolkit of the mouse embryo, including gene knockout (germline or tissue specific) and lineage tracing techniques [3], has kept this species at the forefront of pancreas development studies in the past several decades. Much of our review will therefore focus on mouse development, although we will also discuss insights gained from human embryology and PSC modeling and identify similarities or differences between mouse and human pancreatic development.

The exocrine and endocrine tissues of the pancreas derive from common precursor cells that arise from a dorsal and a ventral domain along the posterior foregut

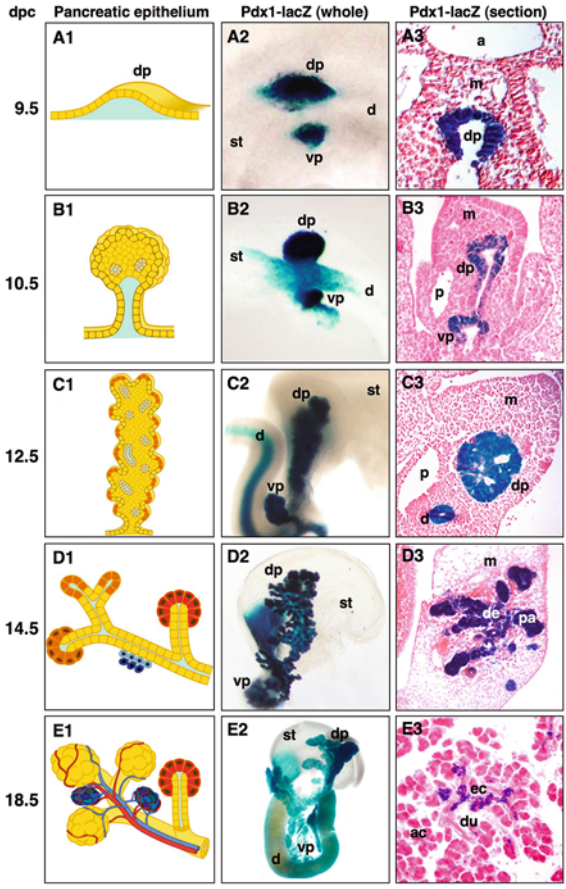

Fig. 1 Overview of pancreatic organogenesis. Schematics and photographs of embryonic pancreas depict development at stages indicated, from (**a**) bud evagination from the endoderm, (**b**) initiation of stratification or branching, (**c**) onset of the secondary transition, (**d**) exocrine and endocrine differentiation, and (**e**) the maturing anatomy of acinar, ductal, and endocrine tissues and associated vasculature just prior to birth. *Left* panels depict the pancreatic epithelium at each stage (mesenchyme not shown). Note the alternative models for dense branching (*left*) versus stratification and microlumen formation (*right*) of the epithelium at 10.5 (B1) and 12.5 dpc (C1). *Yellow*, pancreatic epithelium; *orange*, multipotent precursor cells (MPCs); *red*, differentiating acini; *light blue*, newly emerged endocrine cells; *dark blue*, maturing endocrine cells. *Middle panels* show whole mount views of Pdx1-expressing (*blue stain*) dorsal and ventral pancreatic buds. At 12.5–15.5 dpc, the pancreas is associated with the underlying stomach and duodenum. *Right panels* show sections through Pdx1-expressing epithelium (*blue stain*) surrounded by pancreatic mesenchyme (*pink eosin staining*). *a* aorta, *ac* acini, *d* duodenum, *dp* dorsal pancreas, *du* duct, *ec* endocrine cord, *m* mesenchyme, *p* portal vein, *pa* proacinus, *st* stomach, *te* tubular precursor epithelium, *vp* ventral pancreas

endoderm at the end of gastrulation. The endoderm evaginates (Fig. 1A1–2) at these two sites to form two epithelial buds encased in mesenchyme (Fig. 1A3; mouse 9.5–10 days post-coitum [dpc]; human 25–30 dpc) [4, 5]. The dorsal bud receives important inductive signals, first from the overlying notochord, then from the dorsal

aorta, and finally from the surrounding mesoderm. The ventral bud receives signals from adjacent splanchnic and procardial mesoderm, as well as from the septum transversum. All epithelial tissues of the pancreas derive from these two endodermal buds, which develop further via a dynamic signaling dialogue between the epithelium and the overlying mesenchymes. The dorsal bud generates the gastric and splenic lobes of the murine pancreas, while the ventral bud forms the extensive lobe that runs along the proximal duodenum. In the more compact human pancreas, the dorsal bud forms the head, body, and tail, and the ventral bud forms the uncinate process and inferior part of the head.

Shortly after budding (about 10.5 dpc), the pancreatic epithelium initiates dramatic morphogenetic changes including epithelial stratification and formation of microlumina [5–8] (Fig. 1B1–2). In rodents (but not in humans), the first differentiated endocrine cells appear at this time in the early dorsal bud. The period of bud formation with this early wave of endocrine cells in the rodent pancreas (9.5–10.5 dpc) has been termed the "primary transition" [8]. Slightly later, around 11.5 dpc (35–37 dpc human), both buds have grown and extended, the gut tube turns, and the organ primordia along its axis change their positions relative to each other. At this time, the ventral bud migrates with the bile duct dorsally around the duodenum, resulting in the fusion of the ducts of the dorsal and ventral buds. Whereas the dorsal and ventral pancreases of rodents retain their major ducts, the principal ducts in humans generally fuse to form a main pancreatic duct (of Wirsung) that connects through the ventral pancreas to the common bile duct, while a vestigial accessory duct (of Santorini) maintains its connection to the duodenum through the dorsal pancreas.

By embryonic day 12.5 (40–45 dpc human), cell proliferation has created a densely packed epithelium (Fig. 1C1–3) containing mostly progenitor cells for the islets, acini, and ducts. The number of progenitor cells in the epithelium at this stage determines the eventual size of the mature pancreas [9]. The microlumina have fused to generate a dynamic tubular plexus [10, 11] with second-wave endocrine cell production in the core and acinar cell formation, beginning around the periphery in nascent elongating epithelial tips [10] (Fig. 1C1). The onset of this new striking phase of morphogenesis is termed the "secondary transition" [8]. Subsequently, islet cells arise from regions of the epithelial "trunk" and acinar cells from replicating epithelial cells as the organ continues to expand (Fig. 1D1–3). Pancreatic acini have their own peculiar morphogenesis that is unique among mammalian exocrine glands. The simple tips of the growing epithelium thicken, enlarge, then engulf, and extend over the ends of the tubules, which become intra-acinar intercalated ducts (Fig. 1D1). Consequently, the termini of intercalated ducts extend into the center of the mature acinus [12]. These intra-acinar intercalated duct cells have been called centroacinar cells and have been postulated to possess stem or progenitor cell-like qualities [13].

Select cells within the interior plexus epithelium commit to islet-cell fate and then escape through a process similar to an epithelial-to-mesenchymal-like transition (EMT) [14]. The new islet precursor cells associate along the main pancreatic ducts and in close association with the major pancreatic blood

vessels. Thus, whereas acini and ducts remain within the topological integrity of the tubular epithelium, individual islet precursor cells delaminate from the epithelium (Fig. 1D1). After a short migration and likely still in contact with the underlying epithelium, these islet precursors coalesce into small amorphous cell clusters, reform epithelial contacts, and differentiate into one of five major cell types, each of which express one of the major pancreatic polypeptide hormones (insulin, glucagon, somatostatin, pancreatic polypeptide, or ghrelin).

Prenatal development continues the expansion of the tree-like ductal and acinar tissues and the maturation of islets **(**Fig. 1E1–3**)**. After the secondary transition (in mouse, after ~15.5 dpc), expansion of acinar tissue is predominately through acinar cell replication rather than de novo formation of acini. Extensive acinar cell cytodifferentiation occurs during this period and is marked by the polarization of cells with basal nuclei surrounded by extensive rough endoplasmic reticulum, a highly active Golgi apparatus, and the accumulation of dense secretory (zymogen) granules that fill the entire apical region of the cells. mRNAs encoding approximately 30 digestive enzymes and cofactors rise to very high levels and dominate the total mRNA population [15].

Maturation of the islet cell clusters of rodents occurs progressively, starting with the genesis of endocrine cells at the secondary transition, the gradual coalescence endocrine cells into cords, and the resolution of individual islets during late gestation and in the weeks after birth. The development of mouse islets is characterized by the formation of an α-(glucagon) cell mantle with interspersed δ-(somatostatin), ε-(ghrelin), and PP-(pancreatic polypeptide) cells surrounding a predominately β-(insulin) cell core. Prenatal replication of differentiated endocrine cells is infrequent, and the increase of endocrine tissue during embryogenesis is due almost exclusively to de novo formation from precursor cells in the tubules [11, 16, 17].

Postnatal growth and tissue maintenance occurs principally through proliferation of differentiated endocrine and exocrine cells. Replication of insulin-expressing β-cells begins shortly after birth and gradually decreases. Dividing β-cells are subsequently uncommon but sufficient to maintain β-cell mass [18, 19]. Similarly, acinar cell proliferation decreases postnatally [20, 21] but appears to be the sole source of acinar cell replacement in mature animals [22].

The common origin of islets, ducts, and acini from the duct-like epithelium of the embryonic pancreas underlies an intimate relationship between islets, ducts, and acini in the mature gland [23]. A greater understanding of the development of the endocrine and exocrine compartments, including their structural and physiologic relationships and the principal intrinsic and extrinsic molecular regulators that drive their formation, is important to our growing understanding of the origin and nature of diseases that affect them. Other recent comprehensive reviews: [5, 24, 25]. Here we address current research in the field of pancreatic developmental biology, as these fundamental processes often go awry in pathological conditions such as pancreatic cancer. Key developmental parameters are outlined below in Box 1.

Box 1 Key Research Points

- The acinar, ductal, and endocrine cells of the pancreas derive from a common progenitor cell population that evaginates from the posterior foregut endoderm.
- The budding pancreatic epithelium, encased in mesenchyme, first stratifies, then transforms into a tubular plexus of sufficient early progenitor cells to sustain subsequent development, and establish the final size of the pancreas.
- These multipotent progenitor cells initiate a secondary developmental that generates the acini, ducts, and islets.
- Cell proliferation propels epithelial growth outward, leaving behind precursors for duct and islet cells and forming acini at epithelial tips.
- Islet precursor cells delaminate from central regions of the epithelium, begin endocrine differentiation, and progressively aggregate to form islets.
- The pancreatic program of organogenesis is coordinated by a repeating interplay between extrinsic signals and intrinsic transcriptional regulators.
- Hedgehog, FGF, retinoic acid, Wnt, TGFb, BMP4, Notch, and Hippo cell-cell signaling pathways all contribute to the extrinsic control of pancreatic development.
- Extracellular matrix (ECM), cell adhesion, and integrin-mediated signaling are required for the 3D architecture of the pancreatic epithelium, which is in turn required for proper cell fate acquisition.
- Known pancreas-restricted transcription factors, in intrinsic regulatory networks, specify a pancreatic response to the widely used extrinsic signals.
- In turn, temporal changes in extracellular signals reformulate the transcription factor network in a stepwise manner to resolve cell lineages and control lineage-specific differentiation programs.

Overview of Extrinsic and Intrinsic Developmental Factors

The embryonic formation of all organs is regulated through the stepwise interplay between extracellular developmental signals (generally diffusible extrinsic growth factors, acting as inducers and morphogens) and intracellular mediators of developmental programs (e.g., transcription factors that bind and control specific target genes in a developmental program). Extrinsic factors (EFs) alter the interacting gene regulatory network of intrinsic transcription factors (TFs), which in turn adjusts the developmental state of a cell by changing the pattern of gene expression (Box 2). The induction of new regulatory proteins and the loss of others determine the developmental potential of the cell and its response to subsequent signals. Successive signals during a developmental program transform the transcriptional network of precursor cells in a stepwise manner, increasing cellular differentiation and limiting the developmental options in response to later signals. The program-specific response of a certain cell type to a common signal is

dictated by the nature of the signal and the developmental history of the cell – i.e., its lineage. The record of a cell's lineage is embodied in a particular collection of TFs, the interacting network they create, and their influence on chromatin architecture (Box 3). The network establishes the competence of the cell to respond to a signal and the nature of the response elicited. This chapter will focus on the nature of the extrinsic (intercellular signals) and intrinsic (mostly transcriptional regulatory proteins) factors for pancreatic organogenesis and the developmental processes they control.

Box 2 Extrinsic Developmental Factors (EFs): Cell-Cell Signaling Molecules

Cells and tissues send signals across extracellular space via extrinsic factors. The principal signaling pathways that control organogenesis include TGFbeta/BMP, Notch, Wnt, Hedgehog, receptor tyrosine kinase (FGF, EGF, IGF, and Eph) signaling, nuclear hormone, and JAK/STAT (Fig. 2). Each pathway regulates developmental decisions through the binding of an extracellular factor to a transmembrane receptor on a recipient cell. Binding to the receptor transduces an intracellular response into the recipient cell. The response is propagated as an intracellular signaling event that activates pathway-specific TFs to change gene expression patterns by binding and altering the transcription of a battery of target genes. Myriad extrinsic factors have been demonstrated to control developmental programs. Many of these cell-cell signaling factors have been termed "morphogens," which are secreted into the extracellular space and transmit their developmental effects to nearby cells in a concentration-dependent manner. Cells near a source are exposed to high levels of the morphogen and respond in one way, while cells farther away are exposed to lower levels and may respond differently. Extrinsic developmental factors can also act in a "relay" fashion. For example, a cell that secretes an extrinsic factor may induce a transcriptional response in a nearby responding cell, which reacts by secreting a second extrinsic factor that influences other neighboring cells or the initiating cell, and so on, in a signaling dialogue that alters either the fate of responding cells or their own signaling potential.

Other extrinsic signaling factors are molecules that are tethered to the cell surface and transduce signaling via direct cell-cell or cell-ECM communication. The former category includes cell adhesion molecules, such as adherens junction and tight junction components, which mediate cell-cell adhesion; the latter category includes a wide range of ECM-binding molecules including integrins. A common feature of adhesion modulators is that they are generally tethered to the cytoskeleton and have the ability to transduce a range of signals, from ligand-based to mechanical stimuli.

Extracellular (or extrinsic) signaling molecules are "cell non-autonomous" factors. In other words, they generally regulate genes/responses in recipient cells, rather than in the cells that produce them. "Cell non-autonomy" is a genetic designation indicating the effect of mutations in a gene affects neighboring cells rather than the cells that produce the gene product.

Box 3 Intrinsic Developmental Factors: DNA-Binding Transcription Factors (TFs)
Gene regulatory proteins with the ability to recognize and bind short DNA sequences play the central role in controlling the spatial and temporal transcription of developmentally regulated genes. Once bound to a regulatory site in a promoter or enhancer, these proteins recruit chromatin-modifying enzyme complexes or additional TF complexes that initiate or maintain transcription or, in some instances, do both of these in a stepwise fashion. TFs are often composed of discrete structural domains with specialized functions. A simple DNA-binding TF usually contains a discrete DNA-binding domain, a dimerization domain (TFs often function as homo- or heterodimers), and a transactivation domain (which interacts with the general transcriptional machinery). Approximately 1,300 genes in a typical mammalian genome encode DNA-binding TFs, classified by structural homologies into approximately 30 families of factors. The major families are classified as zinc finger (ZF), basic helix-loop-helix (bHLH), homeodomain (HD), basic leucine zipper (bZip), nuclear receptor (NHR), high-mobility group (HMG-box), Tbox, ETS/IRF, and Forkhead factors. Members of each of these families play prominent regulatory roles in organ development through the genes they bind and control. Many establish the developmental status of cells and determine temporal and stage-specific changes in gene expression in response to extrinsic signaling molecules. Others are the transcriptional effectors of extrinsic signaling pathways.

Remarkably, the genesis of the great diversity of cell types, their integration into distinct complex tissues, and the assembly of tissues into unique organs are directed by a few signaling pathways, each of which is used in the formation of most, if not all, organs. Usually, a single DNA-binding TF (although sometimes a few related factors) specific to a pathway binds target gene promoters and alters their activity in response to the activation of that pathway. There are seven principal developmental signaling pathways, each with their specific transcriptional mediators (Fig. 2).

1. The transforming growth factor-β family (TGFβ/activin/BMP/GDF) pathway with Smad TFs. The TGFβ pathway is generally subdivided TGFβ/activin/Nodal, which use Smads 2 and 3, and BMPs, which use Smads 1, 5, and 8. (Note: the names for genes and proteins are distinguished with italics for genes.)
2. The Hedgehog (HH) pathway with Gli TFs.
3. The Wnt pathway with Lef/Tcf TFs.
4. The Notch pathway with Rbpj.
5. Nuclear hormones with intracellular hybrid receptors.
6. Receptor tyrosine kinase (RTK) pathways with a wide variety of extracellular ligand families (such as fibroblast growth factors (FGFs), epidermal growth factor (EGF), Eph-ephrins, and many more) and downstream transcriptional mediators.

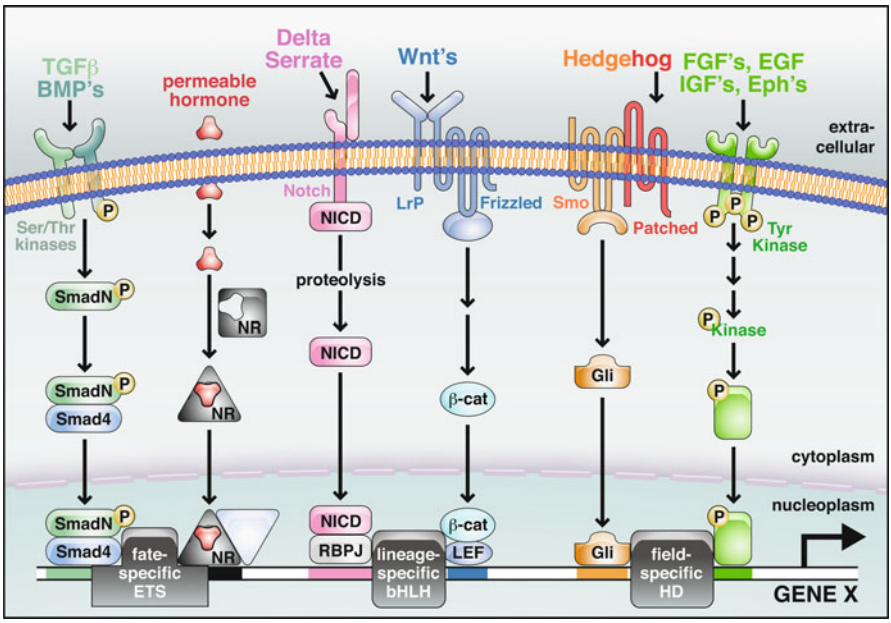

Fig. 2 Major developmental signaling pathways. Pathways from *left* to *right*: TGFβ/BMP, nuclear hormone receptor (with permeable hormones), Notch, Wnt, Hedgehog, and tyrosine kinase receptors for fibroblast growth factors (FGFs), epidermal growth factor (EGF), insulin-like growth factors (IGFs), and Eph/Ephrins. The key to a unique cell-specific outcome (e.g., activation of Gene X) of signaling by a commonly used pathway lies in the developmental history of the responding cell, which is embodied in a specific collection of lineage-specific and spatially restricted transcription factors, such as members of the ETS, bHLH, and homeodomain transcription factor families. Although all pathways discussed in the text are shown, all are not likely to act on the promoter of a single gene

7. The Hippo-Warts pathway, which includes kinases Mst1/Mst2 (hippo) and Lats1/Lats2 (warts) upstream of the transcriptional coactivators Yap and Taz (yorkie orthologues), which bind the transcription factors Tead1/Tead2.

Each of these pathways is critical to proper pancreatic development.

Extrinsic Factors: Cell-Cell Signals

Extrinsic signaling cues regulate multiple aspects of pancreatic organogenesis [26]. These signals are usually transient, are sequential, and frequently have opposing effects at different developmental stages. In particular, several prominent signaling pathways have inhibitory effects on the primary transition and must be kept in check for the pancreatic primordia to be specified [27]. Not only has this phenomenon been observed by experimental embryologists but it is increasingly translated

in efforts to direct the differentiation of PSCs toward pancreatic fates, the protocols for which include application of several pathway inhibitors [28]. Understanding the molecular basis of EF action in the pancreas is a challenge, particularly given the potential for redundancy, the changing responses with age and differentiation state, and the overall cellular complexity of an organ in which multiple cell types engage in paracrine and autocrine interactions. Knowledge of the roles of EFs is important not only for efforts to program pancreatic fates in PSCs but also for understanding pancreatic neoplasia, as most cancers of the pancreas are associated with the dysregulation of these bioactive molecules [29–34].

Here, we introduce those EF signaling pathways known to regulate pancreatic organogenesis. We briefly describe the molecular components that constitute the canonical pathways leading to transcriptional changes in responding cells (Figs. 2 and 4) and provide a specific example for each pathway in pancreatic development.

- Transforming growth factor-β (TGFβ) signaling is based on the binding of secreted extracellular ligands to single-pass transmembrane serine/threonine kinase receptors on responding cells [35]. Ligands in this large family include subfamilies of TGFβs, activins, bone morphogenetic proteins (BMPs), and growth and differentiation factors (GDFs). Ligand-binding induces the heterodimerization of type I and type II receptors. Upon heterodimer formation, the type II receptor phosphorylates and activates the type I receptor, which then transduces the signal by phosphorylating a member of the Smad family of transcription factors. Three types of Smad proteins mediate the transcriptional effects of TGFβ signaling within the responding cell: the receptor-regulated Smads (R-Smads), the common mediator Smads (co-Smads), and the inhibitory Smads (I-Smads). The early specification of the definitive endoderm, from which the pancreas and other gut organs arise, is driven by activin/Nodal-family signaling during gastrulation [36, 37], and PSC differentiation to pancreatic fates requires early exposure to these ligands [28, 38].
- Hedgehogs (HHs) compose a family of secreted signaling proteins that include Sonic (Shh), Indian (Ihh), and Desert (Dhh) hedgehogs, all of which bind a 12-pass receptor subunit called Patched 1 (Ptc 1) [39]. The binding relieves Ptc1-mediated repression of Smoothened (Smo), which is a G-protein-like membrane-associated signaling molecule that transduces intracellular signaling to the nucleus via the Gli family of TFs. Among the earliest detectable steps of pancreas development is downregulation of Shh specifically in the prepancreatic endoderm, induced in the dorsal pancreas by signals from the overlying notochord [40–42]. Loss of Ptc1, resulting in constitutive HH signaling, abolishes pancreas specification. Consistent with a critical role for HH inhibition in early pancreas development, inclusion of the Smo antagonist cyclopamine is essential for directed differentiation of mouse and human PSCs to pancreatic fates [28, 43].
- The Wnts (Wingless/int) are a family of secreted glycoproteins that control cell proliferation, asymmetric cell division, and cell fate [44, 45]. Wnts transduce signaling to responsive cells by binding Frizzled receptors and a variety of co-receptors, such as LRP5/LRP6, RORs1/RORs2, or Ryks. Signaling

downstream of the receptor is transduced via two alternative pathways, roughly categorized as either "canonical" or "noncanonical." The latter includes both the Ca^{2+} signaling pathway (Ca/G-protein/PKC pathway) and the planar cell polarity (or PCP) pathway (frizzled/Rho/JNK). For the canonical pathway, binding of Wnts to a Frizzled/LRP complex leads to stabilization of cytoplasmic β-catenin, which activates target genes by interaction with LEF/TCF family transcription factors. In unstimulated cells, β-catenin levels are kept in check by a cytoplasmic "destruction complex" that includes the proteins APC, Axin, and the serine/threonine kinase GSK3; Wnt binding to Frizzled and LRP5/LRP6 induces inhibition of this complex, allowing accumulation of signaling-competent β-catenin protein. Wnt/β-catenin provides a striking example of the context-dependent actions of EFs in the pancreas, whereas this pathway must be inhibited early; to allow pancreas specification during the primary transition [46, 47], it acts during the secondary transition and after to promote progenitor and acinar cell expansion [47–51].

- The Notch family of receptors mediate juxtacrine signaling, i.e., between immediately adjacent cells [52, 53]. The extremely short range of Notch signaling activity is dictated by an idiosyncrasy of the pathway: both the receptors (Notches) and ligands (Deltas and Jaggeds) are single-pass transmembrane proteins, and activation of the receptor requires active "pulling" by the ligand-producing cell. Intracellular signal transduction is very simple (Fig. 2): after ligand binding and pulling, the Notch receptor is cleaved to release an intracellular portion that enters the nucleus, binds the transcription factor Rbpj, and converts it from a repressor to an activator by recruiting cofactors of the Mastermind-like (MAML) family. Key target genes include the Hes subfamily of bHLH repressor factors, which bind and suppress the transcription of pro-differentiation genes. Notch signaling acts as a binary switch to control two general functions critical to many developmental programs [53]. In some instances, it promotes the expansion of a progenitor cell population by suppressing the decision to begin differentiation; in others, it controls the decision of cells in a population to choose one cell fate at the expense of others. During pancreatic development, Notch signaling performs both developmental functions: it promotes expansion of the progenitor population prior to the secondary transition, while during the secondary transition, it acts in a stepwise fashion to divert cells away from acinar and endocrine fates and toward duct differentiation [54–58].
- Retinoic acid (RA), the active metabolite of vitamin A, binds two types of nuclear receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) or co-receptors, which form heterodimers that translocate to the nucleus to control the transcription of genes containing RA responsive elements (RAREs) [59]. RA is the simplest signaling pathway; its receptor is also the DNA-binding TF that mediates transcriptional control. RA is synthesized from circulating retinol (vitamin A), via an enzymatic pathway including retinaldehyde dehydrogenases (Raldh). Raldh2 is present early and widely during embryonic development [60] and is absolutely required for specification of the dorsal pancreas [61, 62].

Not surprisingly, treatment with RA is a critical step in directed differentiation of PSCs to pancreas [28, 43].

- Receptor tyrosine kinases (RTKs) mediate signaling from numerous families of growth factors, such as FGFs, EGFs, insulin-like growth factors (IGFs), vascular endothelial growth factors (VEGFs), platelet-derived growth factor (PDGF), ephrins, and many others [63]. The cellular outcomes of RTK signaling span a wide range of cell behaviors, including cell proliferation, migration, morphogenesis, cell fate choices, and cell survival. RTKs are single-pass transmembrane receptors, which often hetero- or homodimerize, usually cross phosphorylate each other, and then transduce signaling within the responding cell via multiple pathways, the most prominent of which is the RAS/RAF/extracellular signal-regulated kinase (ERK) cascade. The RTK ligand Fgf10 is particularly critical for pancreas development, being required for proliferation of pancreatic epithelial progenitor cells and maintenance of their organ identity [64, 65].
- The Hippo-Warts signaling pathway controls organ size and is highly conserved from flies to mammals. Key components of this pathway include a kinase signaling cascade, composed of the MST1/MST12 (Hippo orthologues) and LATS1/LATS2 kinases (Warts orthologues), as well as their downstream transcriptional co-activators yes-associated protein (YAP) and its paralog TAZ [66]. Upon phosphorylation, YAP and TAZ are retained in the cytoplasm; however, in the absence of phosphorylation, they are translocated to the nucleus where they interact with the Tea-domain (TEAD) family of transcription factors. Together, YAP/TAZ and TEAD factors stimulate cell proliferation and survival [67]. Deletion of *Mst1/Mst2* in the developing mouse pancreas results in reduced organ mass resulting from postnatal de-differentiation of acinar cells [68]. Ectopic expression of YAP similarly blocks differentiation. Together these findings underline an important role for this family of regulators during pancreas morphogenesis and cell fate lineage allocation.

These extrinsic factors play critical roles in the development of most embryonic organs, including the pancreas, although the effects of any given EF will depend on the developmental status of the recipient cell and may not be consistent throughout organogenesis. As we discuss the different stages of pancreas development in detail, we will review experimental evidence elucidating the diverse roles of the above EFs, including examples where the same factor has seemingly opposite effects.

Intrinsic Factors: DNA-Binding Transcription Factors

The key transcription factors (TFs) that pattern the endoderm, specify and maintain pancreatic fate, and resolve the individual pancreatic cell lineages are known. A model for the pancreatic lineage with associated TFs is shown in Fig. 3. For example, the Forkhead box A2 factor (Foxa2/Hnf3b) controls the formation of the anterior endoderm during gastrulation, the HD protein Mnx1/Hlxb9 participates in endoderm patterning and cell-lineage specification within the pancreatic domain, the

bHLH factor Neurogenin3 (Ngn3) specifies endocrine cell identity, and the bZip proteins MafA and MafB control the final stages of β-cell differentiation. It is important to note that some of these intrinsic factors play critical roles at more than one developmental stage.

In this regard, four TFs merit special mention. The HD factors hepatocyte nuclear factor 1 beta (Hnf1b) and pancreas duodenal homeobox (Pdx1), the bHLH protein pancreas transcription factor 1a (Ptf1a), and the HMG factor Sry-box9 (Sox9) perform distinct regulatory functions at early, middle, and late stages of development. Hnf1b is crucial for essentially every step of pancreas development, including specification of the pre-pancreatic endoderm, growth and branching of the precursor epithelium, development of duct and acinar cells, and initiation of the islet cell genesis [69, 70]. Mouse embryos and human fetuses homozygous deficient for *Pdx1* [71–73] or *Ptf1a* [74–76] do not form a pancreas. Although neither Pdx1 nor Ptf1a is required for the formation of the initial pancreatic buds at 9.5 dpc, both are necessary for the growth, branching morphogenesis, and the transition to the protodifferentiated state. Pdx1 controls the formation and growth of the protodifferentiated cell population; is required at the secondary transition for the formation of the acinar, ductal, and islet cell lineages; and later controls the differentiation and maintenance of β-cells [77–79]. Ptf1a maintains pancreatic identity in the nascent buds, sustains precursor cell growth of the early epithelium, defines the multipotent precursor population that initiates the secondary transition, and later controls the differentiation of acinar cells and maintenance of the mature acinar phenotype [74, 79–82]. Sox9 induces and maintains pancreas identity during organ specification, maintains the undifferentiated state of precursor cells during the primary and secondary transition, and is necessary for proper duct and endocrine cell development [65, 83–86]. As we will discuss, the ability of each of these factors to exert such diverse effects reflects their ability to act in collaboration with additional stage- or cell-specific TFs.

The TFs at the ends of signal transduction pathways (Fig. 2) are the intrinsic mediators of transcriptional control by extrinsic signaling factors. The signaling pathway TFs are thought to collaborate with stage- and lineage-specific TFs in two ways. One is by binding and activating the promoter of a gene encoding a stage- or lineage-specific TF to produce that factor at a specific time and place. The other way is to cooperate with stage- and lineage-specific factors by binding together on the promoter or transcriptional enhancer of a developmentally regulated gene (Fig. 2).

The cooperation of lineage-specific and signaling pathway TFs is the basis for the ability of a signal for a widely used transduction pathway to activate a particular gene in a unique developmental context (Box 4). In general, gene enhancers and promoters require the binding and cooperation of several DNA-binding TFs to be activated. The binding of the transcriptional mediator of a signaling pathway alone is insufficient for activation. This makes sense; otherwise activation of a pathway would induce in a cell the expression of all possible genes regulated by that pathway for all developmental programs. In a complementary fashion, the binding of stage-/lineage-specific transcriptional activators alone is also insufficient. Otherwise, developmental programs would initiate and continue in the absence of extrinsic control

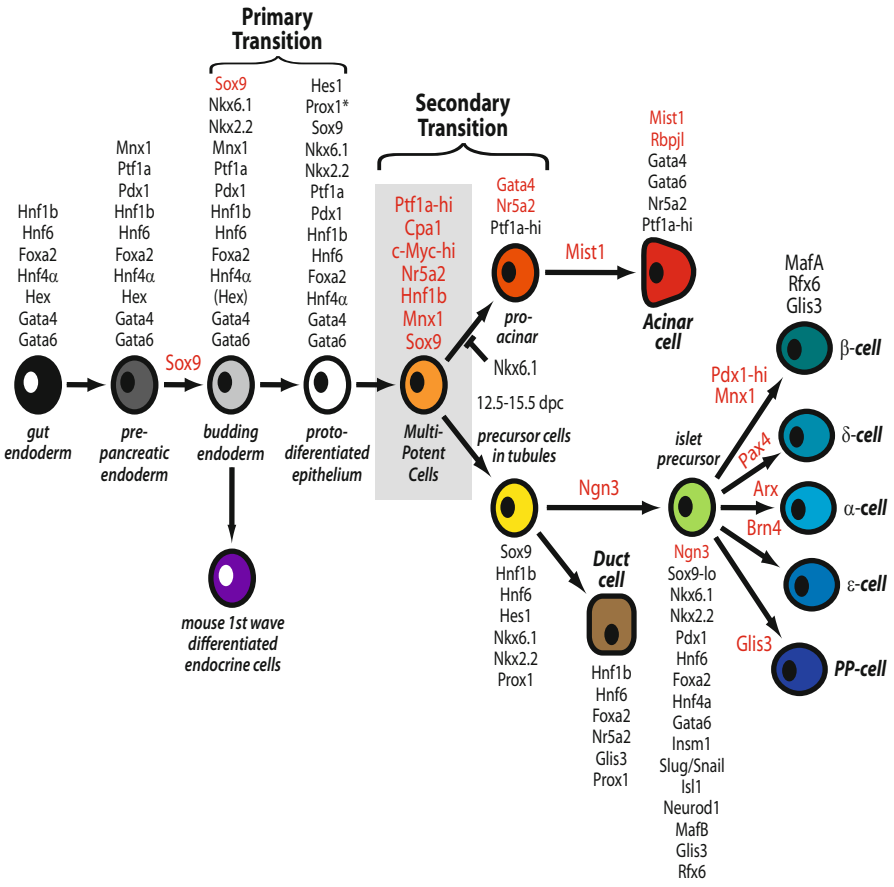

Fig. 3 Primary transcriptional regulators of pancreatic development. Regulatory TFs discussed in the text are listed in association with the progression of cellular commitment and differentiation. Key TFs that appear at a particular stage and likely control the transition to that stage are in *red*. The multipotent precursor cell (MPC) state is highlighted with a *gray* background. The composition of TFs for the first-wave endocrine cells is not listed but is similar to those for the second-wave α- and β-cells. The list of factors is not comprehensive; for example, not all the TFs that distinguish the islet cell types are listed, and some assignments to intermediate cell types are tentative and need confirmation. Prox1*: transient affect on progenitor cells

without regard to correct timing and position in the embryo. Thus, to activate a particular developmentally regulated gene properly, a cell must have the correct history embodied in the presence of the signaling pathway receptor, chromatin markings from a pioneer factor, *and* the appropriate stage-/lineage-specific TFs. The cell also must be in the correct position to receive an effective concentration of the extrinsic signaling molecule released from nearby cells. In turn, for proper regulation, a gene must have a promoter or enhancer with a pre-activation chromatin mark from a pioneer factor and the nucleotide sequences for binding both the

transcriptional mediator of the signaling pathway(s) as well as appropriate stage-/lineage-specific TFs of that developmental program.

Box 4 Extrinsic and Intrinsic Developmental Pathways Converge at the Promoters and Enhancers of Developmentally Regulated Genes

Many, but not all, effects of extrinsic factors on responding cells are transcriptional changes of target genes. The effectors of transcriptional change are the intrinsic, pathway-specific TFs at the end of signaling pathways for extrinsic factors. In many instances, the absence of signaling maintains the pathway-specific TF as a repressor, which keeps target genes firmly off, by recruiting a corepressor [1]. Receipt of a signal converts the transcription factor into an activator, which initiates target gene transcription. For example, in the absence of Wnt ligands, the pathway transcription factor Lef/Tcf is bound to target gene promoters and recruits corepressor proteins that repress transcription. Binding of a Wnt to Frizzled diminishes the destruction of a cytoplasmic structural protein, β-catenin, which then accumulates to a higher level. The increased pool of β-catenin causes some of it to relocate to the nucleus, where it binds Lef/Tcf and either displaces the corepressor or overcomes its action by recruiting coactivators. The change from repression to activation establishes rigorous ON/OFF transcriptional control of target genes necessary for dramatic changes in gene expression.

However, the activation of a pathway-specific TF, such as Lef/Tcf, alone is generally not sufficient to initiate transcription of a developmentally regulated gene. Other TFs already assembled at the promoter are necessary to complement the action of the pathway factor. In this context, the intrinsic lineage- or stage-specific TFs alone are also insufficient to activate transcription. Thus, gene enhancers and promoters with gene-specific combinations of binding sites for pathway-specific and lineage-specific factors act as genetic microprocessors to control developmentally regulated genes (Fig. 2). Thus, the spectrum of genes activated in response to a signal depends on the lineage history of the recipient cell, which is manifested in its set of stage/lineage-specific TFs.

The Roles of Extrinsic and Intrinsic Factors During Pancreatic Development

Here we divide embryonic pancreatic development into four temporal stages and review the roles of extrinsic and intrinsic factors in distinct cellular or morphogenetic events that occur during these stages:

1. Specification of endodermal domains to pancreatic fate (mouse 6.5–9 dpc; human 22–31 days)

2. Initial growth of pancreatic buds and the primary developmental transition (mouse, 9–12 dpc; human, 30–45 days)
3. Onset of acinar, ductal, and islet development by the secondary developmental transition (mouse, 12.5–15.5 dpc; human, 8–18 weeks)
4. Perinatal growth and differentiation (mouse, 16 dpc to neonate; human 20 weeks to neonate)

Specification of Endodermal Domains to Pancreatic Fate

Early Endoderm and Gut Tube Formation

The pancreas forms from the embryonic definitive endoderm, one of three germ layers that emerge during gastrulation (the ectoderm and mesoderm are the others). The ectoderm gives rise to the nervous system and the epidermis; the mesoderm to the muscle, heart, kidney, blood, vasculature, and gut mesenchyme; and the endoderm to the lining of the entire gastrointestinal system, including most organs along its length, such as the pharynx, thyroid, lungs, liver, stomach, pancreas, and intestine. The mouse endoderm emerges from the primitive streak and forms a single epithelial sheet of approximately 500–1,000 cells [87]. As the embryo takes shape, the epithelial sheet rolls up into a primitive gut tube, which runs along the anterior to posterior axis of the embryo. A thick layer of splanchnic mesoderm adheres to the gut tube endoderm during this early phase of morphogenesis, inducing and supporting endodermal proliferation, morphogenesis, and differentiation.

Broad patterning of the definitive endoderm begins as it forms during gastrulation and is based on the timing of the movement of the pre-endodermal epiblast cells through the primitive streak [88, 89]. The first presumptive endodermal cells exiting the primitive streak become the most-anterior and most-posterior endoderm, followed by cells that form the middle endoderm and the rest of the posterior endoderm. During the passage of cells through the primitive streak, signaling by Nodal (a member of the extended TGFβ/BMP family of morphogens) preferentially establishes the anterior foregut endoderm in part through the induction of Foxa2, a Forkhead TF also important for subsequent endodermal organogenesis [90]. Embryos deficient in Smad2, a TF mediator specific to the TGFβ/activin/nodal subfamily of extrinsic signals, fail to generate endoderm properly [36]. This pathway of definitive endoderm induction was first described in fish and frog [91], and its deep conservation across vertebrates is highlighted by the fact that induction of definitive endoderm from human pluripotent stem cells (PSCs) requires treatment with Nodal or the related Smad2-/Smad3-activating ligand activin [28].

Anteroposterior Patterning of the Endoderm

The broad developmental domains of the early definitive endoderm resolve progressively to form the pharynx, esophagus, stomach, intestine and colon, and the glands that bud off the gut tube during organogenesis (the submandibular and sublingual glands, thyroid, parathyroid, trachea, lungs, liver, and pancreas). Although the early

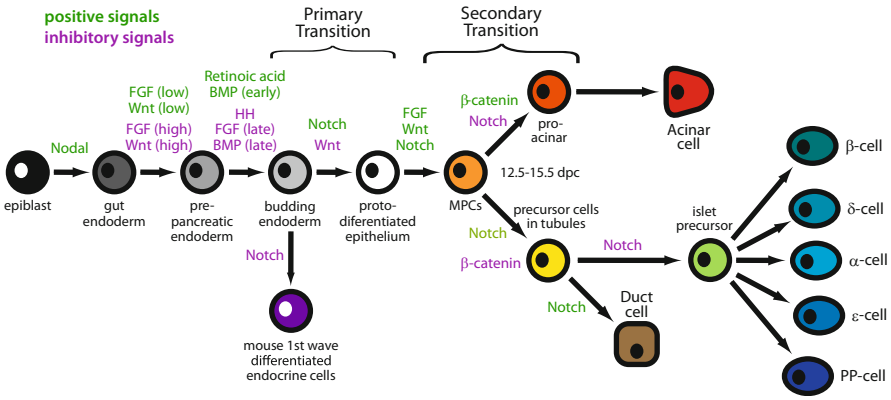

Fig. 4 Cell-cell signaling during pancreatic development. The lineages of the developing pancreas are depicted as in Fig. 3. Key signaling factors that control specific developmental transitions are indicated in *green* or *purple*, according to whether they promote or inhibit those transitions. Notably, the direction of a given signal can change dramatically at different times and with different concentrations. For example, canonical Wnt signaling is required at low levels for the specification of the pre-pancreatic endoderm but inhibitory at high levels. Wnt must thereafter be downregulated to allow the primary transition to occur but then is reactivated to promote proliferation of MPCs

endoderm appears morphologically homogeneous prior to the onset of organogenesis, it is in fact patterned along its anteroposterior axis.

How is the broad anteroposterior regionalization of the nascent endoderm refined? The regional expression of lineage-specific intrinsic factors is established through complex, extrinsic signaling from the mesoderm to the underlying endoderm and back again [91]. For example, the endoderm is patterned in a concentration-dependent manner by FGF and Wnt signals produced by adjacent mesoderm (Fig. 4). In experiments with mouse and chick embryos, using explant culture and bead implantation, exposure to high levels of FGF4 promotes posterior (intestinal/hindgut) fate, whereas lower levels allow more anterior cell fates including *Pdx1*-expressing pancreas [92, 93]. This extrinsic FGF signal acts directly on cells of the endoderm (rather than indirectly via the mesoderm), as expression of a constitutively active FGF receptor (FGFR1) in the endoderm also leads to the same anterior expansion of *Pdx1* expression. The posterior mesoderm also appears to be a source of Wnt ligands that promote intestinal (hindgut) development while repressing foregut fates including pancreas and liver. This is revealed in knockdown and overexpression experiments targeting the canonical β-catenin pathway in zebra fish and frog [94, 95]. Interestingly, directed differentiation experiments in human PSCs indicate that the effects of Wnts on endoderm patterning, similar to those of FGF, are concentration dependent, with low levels actually enhancing pancreas fate specification while high levels inhibit it [96].

Several transcription factors with restricted expression cooperate with developmental signaling to control regional identity along the endoderm. The foregut

endoderm expresses regulators of anterior developmental programs not found in posterior endoderm, such as the HMG-box factor Sox2 and the HD proteins Six, Nkx2.1, and Hex. Six and its coactivator Eya1 pattern a subregion of the pharyngeal endoderm for thyroid and parathyroid formation [97]. Sox2 and the HD factor Nkx2.1 play reciprocal roles in resolving the esophagus and trachea: Sox2-deficient esophageal endoderm acquires a tracheal phenotype including ectopic *Nkx2.1* expression, and Nkx2.1-deficient tracheal endoderm initiates a partial esophageal developmental program, including ectopic *Sox2* expression [98, 99]. The HD factor Pdx1 is first restricted to the initial domains of the prepancreatic buds and then expands to include the proximal duodenum and the distal stomach [72]. The HD factors Cdx1 and Cdx2 establish the intestinal region of the gut tube distinct from the stomach and more anterior regions and are excluded from the pancreatic domain [100, 101].

Initiation of Pancreatic Fate and Morphogenesis

Soon after early endodermal gut tube formation, the first morphological sign of pancreatic development is a local thickening and evagination of the dorsal midline endoderm at about E8.75 in the mouse and during the fourth week of gestation in humans. Cells within the thickening epithelium change from cuboidal to columnar, which drives the growth of a small fin-like evagination. Approximately 12 h later, as the anterior intestinal portal closes over the pancreatic domain, the ventral pancreatic evagination becomes evident. The dorsal pancreatic bud emerges just caudal to the developing stomach, and the ventral bud appears just caudal to the developing liver, near the base of the primordium of the common bile duct. Some mammals are thought to form a single ventral bud (rat and human), whereas others have two clear ventral buds (frogs and chick). In mouse, a second ventral bud is present transiently [40, 102].

These morphological changes are prefigured by changes in gene expression, in particular the downregulation of *Shh* and upregulation of *Pdx1* within the future dorsal and ventral pancreatic buds [40, 42, 103, 104]. Although *Pdx1* itself is required for the outgrowth of the pancreatic epithelium after budding, rather than budding itself [72], its early expression provides a convenient marker for the process of pancreas specification. Several TFs and EFs have been found to be essential for the initial events of pancreas specification, in several cases acting differently between the dorsal and ventral primordia. The SRY-box TF *Sox17* and the homeodomain TF *Hnf1b* are required for both pancreatic buds, with defects manifesting at the initial stages of *Pdx1* expression [105, 106]. By contrast, the homeodomain TF *Mnx1/Hlxb9* and the Zn-finger TF *Gata4* are required selectively for specification of the dorsal and ventral pancreata, respectively [107, 108]. Similarly, the earliest requirement for the bHLH TF *Ptf1a*, which plays multiple roles in pancreas development, appears to be the determination of ventral *Pdx1*+ endodermal cells to a pancreatic fate; in its absence, these cells are respecified to the duodenum and bile duct, "next-door neighbor" organs relative to the ventral pancreas [74, 79]. Reciprocally, misexpression of *Ptf1a* is sufficient to induce *Pdx1* expression, and

ectopic pancreas development, from endodermal tissue outside the normal pancreatic domain [80, 109].

While it is not yet clear why different TFs are required for initiation of the dorsal and ventral buds, it is possible that these factors act in collaboration with extrinsic factors whose activity differs between dorsal and ventral endoderm. For example, chick and mouse studies have identified the notochord and dorsal aorta as providing critical early cues for dorsal, but not ventral, pancreas specification [40, 41, 110, 111]. Shortly after these interactions, the lateral plate mesoderm migrates around the endoderm to provide additional signals for dorsal bud formation. Among these is retinoic acid, synthesized by mesodermal Raldh2 and required for dorsal, but not ventral, bud development [61, 62]. Interestingly, retinoic acid treatment is an essential step in the directed pancreatic differentiation of human pluripotent stem cells, indicating that this process may model dorsal bud development specifically [28, 43]. Dorsal pancreas agenesis is also observed in *Isl1* mutant mice, reflecting a cell non-autonomous requirement for this TF in the lateral plate mesoderm [112]. By contrast, initiation of the ventral, but not dorsal, pancreas depends on signaling through Smad4, mediating a narrow window of pro-pancreatic BMP signaling [113].

In addition to positive signals that promote its development, the pre-pancreatic endoderm is vulnerable to an array of inhibitory signals that must be evaded for specification to occur. Prominent among these is the HH pathway, downregulation of which is obligatory for pancreas development in vivo [41], as well as elaboration of pancreatic cell types from human PSCs in vitro [28, 43]. BMP and FGF signaling, which have positive roles in pancreas specification as noted above, are also major negative regulators of ventral pancreas specification. Emanating from the precardiac mesoderm and septum transversum, these signals promote liver development at the expense of pancreas [114, 115]. If cells fated to the ventral pancreas are unable to move away from these influences, as occurs in mice lacking the endodermal TF *Hex*, they are diverted to a liver fate [116]. The remarkable changes in the endodermal response to FGF and BMP signaling [113] are not unique to these pathways, or to early specification; as we will see, analogously variable response to Wnt and Notch signaling is observed at later stages of pancreas development. Indeed, a great deal of current efforts in the field of pancreatic studies involves elucidating the sequential exposure of pancreatic progenitors to extrinsic positive and negative cues, including WNT, FGF, and Notch signaling and their influence on cell lineage allocation (Fig. 4).

Initial Growth of Pancreatic Buds and the Primary Developmental Transition (9–12 dpc)

The early phase of pancreatic development, or the "primary transition" (Fig. 5), involves growth of the epithelium, the appearance of a few differentiated "first-wave" endocrine cells in rodents, and the formation of a lumen. The nascent bud first forms a complex, stratified epithelium containing a pool of progenitor cells of

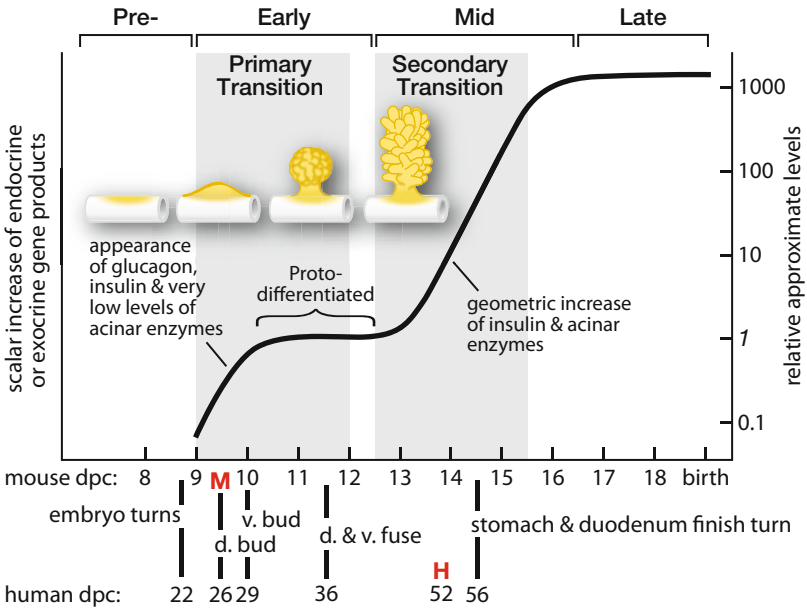

Fig. 5 The primary and secondary transitions of pancreatic development. The primary and secondary transitions were originally defined by William Rutter, Raymond Pictet, and their colleagues on morphologic criteria [8] and biochemical quantification of the products made by differentiated endocrine and exocrine cells [157, 267, 268]. The primary developmental transition marks the appearance of very low levels of acinar digestive enzymes (principally Cpa1) and the first-wave glucagon-gene, and subsequently insulin-gene, expressing cells. The secondary developmental transition spans the geometric increase of acinar digestive enzymes and insulin. Human pancreatic development has a primary transition that forms a protodifferentiated epithelium, but not first-wave endocrine cells. Human development also has a secondary transition stage, but it does not begin at the same time in all regions of the larger pancreatic rudiment and so appears much less concerted than the rodent transition [269]. The first endocrine cells appear in the primary transition at 9 dpc in mouse embryos (*red* M) and at about 50 dpc during the extended secondary transition in human fetuses (*red* H)

sufficient size to allow the proper transformation of the pancreas into a tubular tree-like organ at the "secondary transition," with its array of islet, ductal, and acinar tissues. Here, we describe the cellular events that define the developmental progression of the dorsal pancreatic epithelium during the primary transition. Interestingly, within the last half decade, improved imaging and immunofluorescent techniques have significantly advanced our understanding of pancreatic morphology and development, allowing unprecedented elucidation of cellular events during pancreatic bud ontogeny.

Epithelial Microlumen Formation

Prior to budding, the endoderm destined for dorsal pancreas transforms from a flat, thin, and simple cuboidal epithelium (Fig. 1a) to a thickened columnar epithelium that begins to acquire multiple layers [10]. It is this localized growth that initiates

budding of the epithelium into a fin-like structure. As growth continues, the neck of the bud constricts and the bud takes on a fist-like appearance, containing a compact epithelium surrounded by mesenchyme. During this externally visible bud development, dramatic changes are occurring within the epithelium. Indeed, it was noted over a decade ago that small isolated lumens, termed microlumens, opened between cell layers, which were proposed to constitute an initial event in the formation of branches [7]. This epithelial mechanism for tubular network formation is also found in the development of the exocrine pancreas of zebra fish [6]. In this species, the branched ductal epithelium arises from the formation of microlumens within a stratified epithelium, and their subsequent fusion creates the branching ductal tree. Recent studies have now demonstrated that microlumen fusion initiates pancreatic branching in mammals as well [10, 11, 117].

Ductal Plexus Formation

The appearance and interconnection of microlumens within the epithelium rapidly form a three-dimensional network of ductal tubules (Fig. 6). Recent work has shown that this plexus forms in the stratified region of the developing pancreas epithelium, referred to as "trunk" or "body" cells [10, 24, 118]. This region has further been identified as the niche where endocrine progenitors later arise [11]. Disruption of expression of the endocrine progenitor factor Neurog3 or inhibition of Notch signaling results in defects in both ductal plexus remodeling and differentiation of endocrine cells. Interestingly, ablation of *Pdx1*, which has long been known to be

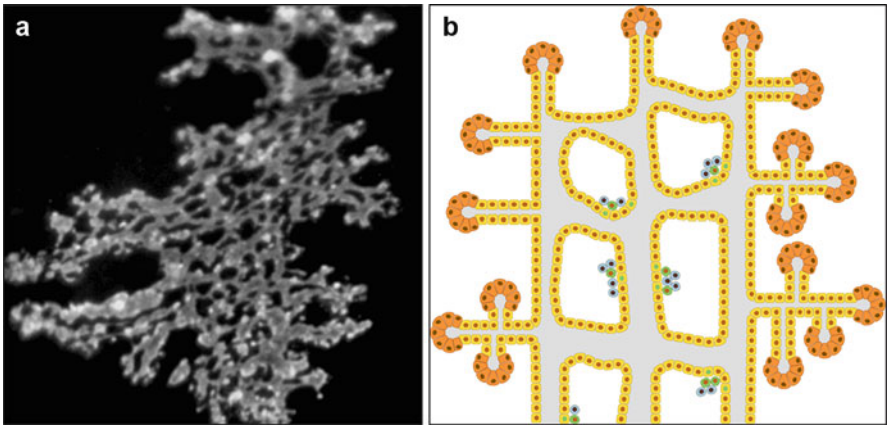

Fig. 6 At the cord of the midgestation embryonic pancreas is a transient ductal plexus, where endocrine cells are generated. Microlumina form within the stratified epithelium of the early pancreatic bud, which then fuse into a 3D ductal plexus. In the core region of this plexus, endocrine progenitor cells express Ngn3 and then delaminate from the epithelium to generate endocrine lineages. *Yellow*, precursors in the tubules for duct and islet cells. *Orange*, pro-acinar tip cells. *Green nuclei,* scattered cells in the tubules initiate *Ngn3* expression. *Green cells* have escaped the epithelium and begun the islet cell developmental program. *Light blue*, differentiating endocrine cells that have initiated the synthesis of an islet hormone. *Gray*, epithelial lumen cavity

required for both acinar and endocrine fate in the pancreas, also results in significant defects in the pancreatic epithelial architecture, with complete failure in the maintenance of the fine plexus of ductal lumens and reduction of E-cadherin levels [119]. Hence, epithelial architecture has been proposed to be intimately linked to differentiation of pancreatic lineages, including both endocrine and exocrine lineages.

Epithelial Reorganization and Regionalization

The defining characteristics of ductal plexus formation are the initiation of regionalized and distinct changes of cell shape and behavior. While microlumens are forming and connecting within the bud core, cells at the periphery, termed "cap cells," acquire a distinctly columnar appearance [10]. The cap cells are both $Sox9^+$ and $Ptf1a^+$ and become allocated to "tip" domains and ultimately an acinar fate. Cells within the trunk-like interior epithelium are $Nkx6.1^+$, $Sox9^+$, and $Hnf1b^+$ and constitute bipotent ducto-endocrine progenitors [120]. Shih and colleagues find that cap cell specification is induced by ECM-integrin activation of FAK/Src signaling, which controls actomyosin and suppresses E-cadherin adhesion [118]. These changes in cell adhesion and morphology result in differential cell behavior and branching of outer versus inner cells. Loss of β1-integrin leads to loss of cap cell segregation and branching morphogenesis. Live imaging of the normal developing pancreatic bud showed that cap cells exhibit mitosis-associated cell dispersal involving release from the epithelium, division, and reinsertion at distant locations. This process has been observed in other epithelial organ systems such as the kidney tubular epithelium and described as "luminal mitosis" [121]. A better understanding of cell shape changes, and motility during the stages of pancreatic morphogenesis will help us characterize the epithelial niche for endocrine cell differentiation.

The Protodifferentiated State

The epithelial cells of the nascent buds are specified to begin the pancreatic program and transition to a committed protodifferentiated state characterized by low level expression of acinar gene products (Fig. 5); a small number of differentiated endocrine cells appear at this stage [8, 122]. Referred to in rodents as the "primary transition," this shift toward a characteristic pancreatic pattern of gene expression reflects the upregulation of a battery of transcription factor genes, including *Pdx1*, *Ptf1a*, *Sox9*, *Nkx6.1*, and *Hnf1b*, that regulate both progenitor and differentiated cell-specific genes. As described below, this phase of pancreatic development is characterized by critical interactions with surrounding mesenchyme that drives both outgrowth and differentiation. It is important to note that pancreatic identity is not irrevocably fixed at these stages, as evidenced by studies in which abnormal signaling or transcriptional processes can cause respecification of the pancreatic buds. For example, hyperactivation of Wnt/β-catenin signaling in Pdx1+ cells induces cystic, gut-like structures devoid of pancreatic gene expression [47]. A very similar phenotype occurs in mice lacking the HD TF Bapx1, which is normally required for separation of the pancreatic and splenic mesenchyme; when the splenic mesenchyme remains adjacent to the dorsal pancreatic bud, it induces respecification

to a gut-like fate [123]. By contrast, loss of the epithelial TF Sox9 causes cells in both the dorsal and ventral pancreata to undergo hepatocyte differentiation, beginning after bud outgrowth [65]. Therefore, although we follow the usual convention of describing distinct phases of pancreas development, it is important to remember that the underlying processes regulating specification, growth, and differentiation are likely to occur in overlapping windows of time.

First Wave Endocrine Cells: Glucagon Cells Bud from the Epithelium

The first endocrine cells in rodents appear during the early stages of bud evagination (9 dpc in mouse), either as single cells integrated in the epithelium or clusters of cells that remain attached to the pancreatic epithelium [124]. Initially, all of these early endocrine cells express the glucagon gene, and as the number increases, a few cells co-express glucagon and insulin, and later some only insulin, although the majority express glucagon only. These observations initially suggested that insulin-expressing cells throughout development might derive from glucagon-expressing cells, through an intermediate co-expressing both hormones. Lineage tracing and ablation studies, however, demonstrated that mature β-cells and α-cells did not develop from precursors with overlapping insulin/glucagon expression [125, 126]. The ultimate fate of these enigmatic "first-wave" endocrine cells remains unproven, and the absence of a homologous population in early human pancreas development [127, 128] makes their relevance to human pancreatic development uncertain. Nonetheless, insulin/glucagon double-positive cells are frequently generated in protocols for directed differentiation of human pluripotent stem cells and are generally regarded as an undesirable, nonfunctional byproduct of these techniques [28].

"Founder Cells" and Early Determination of Organ Size

The majority of cells during the protodifferentiated stage are multipotent progenitor cells (MPCs). These express transcription factors including Pdx1, Ptf1a, and Sox9 that are collectively required to expand the MPC population to the size needed at the secondary transition to generate the proper number of differentiated acinar, ductal, and islet cells. Interestingly, the size of the early MPC population ultimately determines the final size of the organ. This is indicated by experimental ablation studies, in which cell-autonomous expression of a toxic transgene is used to kill a subset of the protodifferentiated cell population [9]. Experimental ablation of a fraction of pancreatic progenitor cells prior to 9.5 dpc has little or no effect on final organ size. By contrast, ablation of progenitors during the phase of protodifferentiated cell expansion (9.5–12.5 dpc) limits the size of the pancreas at birth and in adulthood in proportion to the number of lost progenitors. This reduction in size affects both endocrine and exocrine cells. Thus, pancreatic size is dependent on the number of MPCs established prior to the secondary transition and is largely independent of regulatory influences that might modulate this population during subsequent growth and development. By analogy to "founder effects" in human populations, in which impacts on a small number of ancestors reverberates to their descendants, mutations that cause agenesis or hypoplasia of the mature pancreas are likely to reflect very

early impacts on the establishment, growth, or survival of the protodifferentiated population.

Epithelial-Mesenchymal Crosstalk: Control of the Protodifferentiated State

Growth and differentiation of the pancreatic epithelium requires critical signals from the surrounding mesoderm. This was demonstrated decades ago in elegant embryological recombination experiments [129, 130]. When cultured as an intact rudiment including both endoderm and mesoderm, the protodifferentiated pancreatic bud undergoes growth as well as differentiation, into both endocrine and exocrine cells. Removal of the mesenchyme dramatically impairs epithelial growth and biases differentiation dramatically away from exocrine and toward endocrine cells [130–132]. In vivo, ablation of the mesenchyme by expression of a toxic transgene product causes pancreatic agenesis, likely resulting from defective MPC growth or survival [133]. Although early studies indicated that at least some of the effects of the pancreatic mesenchyme could be recapitulated by a partially purified factor [134], the nature of the signal that emanates from the mesenchyme has yet to be fully resolved, almost certainly because it comprises multiple molecular species that act both separately and in cooperation.

For example, as described above, recent work points toward a key role for extracellular matrix and cell-cell adhesion molecules in partitioning protodifferentiated cells between "cap" and "body" populations [10, 118]. Cap cells are polarized by contact with basement membrane molecules synthesized by surrounding mesenchyme cells, including laminin. Laminin has the intriguing property of being required for exocrine (acinar and duct) development in cultured pancreatic buds, while suppressing endocrine differentiation [117, 135]. These studies indicate that the physical proximity of epithelium and mesenchyme is as important as any diffusible signaling factors transferred between these tissues.

Nonetheless, secreted factors from the mesenchyme are essential for proper pancreatic development, most prominently FGF signals that promote expansion of the protodifferentiated cell population. Mouse embryos lacking the FGF receptor 2b (FGFR2b) or expressing a dominant negative form develop acute hypoplasia affecting both exocrine and endocrine lineages [136, 137]. FGF10, a ligand for FGFR2b, is expressed by pancreatic mesenchyme and required early (10.0–12.5 dpc) for proper pancreatic budding and growth. Loss of *Fgf10* function eliminates the expansion of the progenitor cell pool but not the specification of the first-wave endocrine cells [64]. FGF10 is also required to maintain Sox9 expression in the epithelium and, thereby, prevent respecification of protodifferentiated cells to a hepatic fate [65]. The importance of FGF signaling to pancreas development is also demonstrated by directed differentiation studies in pluripotent stem cells, in which inclusion of FGF10 or the related Fgfr2 ligand FGF7/KGF during pancreas specification steps dramatically enhances the generation of MPC-like cells [43, 138]. As in the earlier stages of specification, FGF signaling has dose-

dependent effects at the protodifferentiated stage: Fgf10 overexpression in the pancreatic endoderm leads to marked hyperplasia, prolonged maintenance of *Pdx1* expression, and suppression of pancreatic endocrine and exocrine differentiation [139, 140]. This effect appears to be partly due to the dysregulation of the Notch pathway.

Notch signaling is known principally for its regulation of binary cell fate decisions, a process referred to as "lateral inhibition" or "lateral specification" in which neighboring cells parse out their respective fates by reciprocal signaling [52, 53]. In this process, a ligand (e.g., Delta or Jagged) produced under the direction of a transcriptional regulator (e.g., Ngn3, in the embryonic pancreas) binds and activates a cell-surface Notch receptor on a neighboring cell, which, in turn, activates a transcriptional response through the downstream transcription factor Rbpj (Fig. 2). The key element of the response is the induction of *Hes1* or related members of the *Hes* gene family within the cell bearing Notch receptors. In mutant mouse embryos lacking the Notch ligand Dll1, the Notch partner TF Rbpj, or the downstream target Hes1 in the pancreatic epithelium, the protodifferentiated cell population is not maintained [55–57, 141, 142]. The Hes TFs are transcriptional repressors that inhibit the expression of pro-endocrine factors such as Ngn3 in receiving cells. In the pancreatic epithelium prior to 12.5 dpc, this suppression of differentiation promotes the expansion of the protodifferentiated cell population. Loss of function of Notch pathway genes at this stage of development leads to the uncontested expression of *Ngn3* and to the premature differentiation of MPCs into glucagon-expressing first-wave endocrine cells [55, 56].

As mentioned above, the maintenance of the protodifferentiated state MPC by Notch signaling is itself affected by extrinsic factors, such as Fgf10, from the mesenchyme. Forced expression of Fgf10 in the early pancreatic epithelium causes the inappropriate high-level expression of the Notch ligands Jagged1 and Jagged2, which leads to the persistent induction of Notch receptors and Hes1 [57, 139, 140]. The superinduction of Hes1 and possibly other Hes family members suppresses differentiation, at least in part, by repressing *Ngn3* expression, and promotes cell proliferation. This cascade of effects suggests that pancreatic mesenchyme normally promotes acinar and beta cell development indirectly by extending the window of epithelial Notch signaling via FGF10, thus allowing the protodifferentiated progenitor pool of the epithelium to expand [64]. Overall, however, the epithelial defects observed in the absence of *Fgf10* mutants are less severe than those caused by complete lack of mesenchyme; for example, exocrine tissue still develops in *Fgf10* mutant pancreata, albeit at reduced overall mass [64]. In addition, the endocrine-suppressive effects of this tissue cannot be recapitulated in vitro by treatment with FGF10 [143], indicating that multiple additional signals must be sent by the mesenchyme to promote MPC expansion.

Within the epithelium itself, as noted, Notch signaling appears to be a major driver of MPC expansion [51, 57]. Emerging evidence implicates the Wnt signaling pathway as another pro-proliferative cue, although its effects on differentiation are more complex. Deletion of β-catenin (*Ctnnb1*), the key mediator of canonical Wnt signaling, causes reduced proliferation of protodifferentiated cells and overall

pancreatic hypoplasia [48, 50]. The epithelium itself abundantly expresses the Wnt ligand *Wnt7b*, deletion of which also causes pancreatic hypoplasia [51]. The *Wnt7b* hypoplasia phenotype is less severe than that of β-catenin knockouts; one explanation for this difference is that other Wnt ligands are expressed and partially redundant with Wnt7b. However, β-catenin mutants also exhibit patterning and differentiation defects not seen in *Wnt7b* mutants, including loss of acinar differentiation (see below); these might reflect Wnt-independent roles of β-catenin in cell-cell adhesion, consistent with recent studies emphasizing the importance of adhesive cues in the early pancreas [118, 119].

These extrinsic factors that promote pancreatic bud outgrowth must act in cooperation with key transcriptional regulators, including Sox9, Pdx1, Ptf1a, and Hnf1b, active in the protodifferentiated epithelium and required for its expansion (Fig. 3). The HMG-box transcription factor Sox9, for example, controls a transcription network [144] that sustains the precursor cell population by deferring differentiation while promoting cell proliferation and survival [85]. Developmental abnormalities in Sox9-haploinsufficient human fetuses are consistent with an inability to sustain a proper pancreatic progenitor population during pancreatic organogenesis [145]. Elimination of Sox9 in the developing pancreas causes failure to maintain the pool of protodifferentiated precursor cells due to decreased cell proliferation, increased apoptosis, diversion of cells to differentiation to the early endocrine lineage of glucagon-expressing cells [85], and fate conversion to the liver lineage [65]. As described above, Sox9 is also involved in FGF signaling within protodifferentiated cells by driving expression of the Fgfr2b receptor, which establishes a reinforcing regulatory circuit that maintains pancreatic progenitor cells as long as Fgf10 is produced by the mesenchyme [65].

Experimental manipulation of embryonic *Pdx1* expression in utero was used to show that *Pdx1* is also required for the expansion of the protodifferentiated epithelium and its subsequent differentiation [77]. Depletion of Pdx1 during the protodifferentiated stage (9.5–12.5 dpc) inhibited cell proliferation (Hale and R.J.M unpublished). Depletion at progressively later developmental times allowed incremental expansion of the protodifferentiated epithelium and thereby further pancreatic growth and development. For example, the depletion of Pdx1 after 12.5 dpc allows some acinar and islet development. The complete absence of *Pdx1* results in pancreatic agenesis in mouse and human, due to arrest and malformation of the pancreas at the protodifferentiated stage [72, 73, 119, 146]; this requirement is recapitulated in human PSCs, in which engineered deletion of *Pdx1* completely blocks genesis of pancreatic cell types [147].

The expression of the bHLH factor Ptf1a begins in the epithelium of the nascent pancreatic bud, expands throughout exocrine and endocrine cell progenitors of the primary transition, slowly wanes during the protodifferentiated state, and is reestablished prior to the secondary transition [74, 79, 148, 149]. Ptf1a is necessary for the formation of the ventral pancreatic bud and for the proper growth and development of the dorsal bud [74, 75, 149]. In the absence of Ptf1a, the protodifferentiated cell population does not expand; consequently, the secondary transition does not occur, and only an incomplete main pancreatic duct forms.

Experiments with both frogs and mice in which *Ptf1a* was inappropriately expressed in the early endoderm demonstrated the potential of *Ptf1a* to specify pancreatic fate at ectopic sites in the embryo [80]. The ectopic expression of *Ptf1a* converted the anterior duodenum, the extrahepatic biliary system, and the glandular stomach to pancreatic tissue, including acini, ducts, and islet-like endocrine cell clusters. Thus, the normally precise expression of *Ptf1a* at specific regions of the endoderm positionally restricts pancreas formation and prevents the disruption of other foregut organs. Interestingly, although mutation of *Ptf1a* in humans causes pancreatic agenesis, its deletion in PSCs does not prevent generation of pancreatic endoderm in tissue culture [76, 147]. This may reflect a role for Ptf1a in inhibiting signals that normally suppress pancreas development in vivo (Fig. 4), which may not be present in vitro.

bHLH transcription factors like Ptf1a generally act as homo- or heterodimers that bind a six-base pair DNA recognition sequence. Ptf1a is the only bHLH factor known that requires a third DNA-binding subunit (either Rbpj or Rbpjl), which extends its functional binding site to 21 base pairs [150, 151]. A single tryptophan-to-alanine substitution near the carboxyl terminus of Ptf1a disrupts the ability of Ptf1a to recruit Rbpj (but not Rbpjl) into the trimeric complex. The extensive developmental defects of *Ptf1a*-null embryos are recapitulated in embryos homozygous for this single amino acid change [149]. Thus, the biochemical form of Ptf1a required for the early stages of pancreatic development is the trimeric complex including Rbpj and called PTF1-J. This developmental role for Rbpj is distinct from its role in Notch signaling. Whereas its function as part of the Notch-pathway is to prolong the protodifferentiated state by preventing cellular differentiation, its function as a subunit of the PTF1 complex is to sustain the developmental program of the early epithelium [149].

Hnf1b is another intrinsic factor required for the protodifferentiated state and the expansion of pancreatic progenitor cells. The results from lineage tracing showed that *Hnf1b*-expressing cells of the early rudiment contribute extensively to all three epithelial lineages [69]. In the absence of Hnf1b, the ventral pancreatic bud does not form; however, the dorsal bud forms, begins normal growth, and then fails to expand the protodifferentiated cell population effectively [105]. The absence of Hnf1b leads to decreased proliferation and increased death within the *Pdx1*-expressing progenitor population [70]. The developmental actions are mediated through Hnf6/Onecut1, Pdx1, and Ptf1a [105, 152]. Indeed, the developmental phenotype is similar to that of *Ptf1a*-deficient embryos [74, 149] and the depletion of Pdx1 after initial pancreatic bud formation [77].

Gata4 and *Gata6* have essential and partly redundant functions in pancreatic development. Whereas the absence of either Gata4 or Gata6 modestly affects the formation of the exocrine compartment (see below), the absence of both leads to early developmental failure of the pancreatic buds [153, 154]. The inactivation of *Gata4/Gata6* at the onset of pancreatic development allows the formation of the initial buds, but *Shh* and other genes of the Hedgehog signaling pathway are activated, and the cells of the dorsal and ventral pancreas convert to lineages of the stomach and intestine, respectively [155].

Onset of Islet and Acinar Development by the Secondary Developmental Transition (12.5–15.5 dpc)

The next stage of pancreatic organogenesis converts the protodifferentiated epithelium of expanding progenitor cells into a dynamic epithelium that generates acinar cells, differentiated ductal cells, and the second (principal) wave of endocrine cells that form the islets. This dramatic and critical conversion period is termed the "secondary transition" (Fig. 5). It was recognized initially by the sudden appearance of large numbers of insulin-producing β-cells [8], the expansion of the glucagon-producing α-cell population [156], and the appearance of pro-acini coincident with a massive increase in the synthesis of acinar digestive enzymes [157]. Highly proliferative cells in epithelial tips around the periphery of the pancreatic rudiment form a domain of rapid outward growth [158]. By the end of the secondary transition, a greatly expanded and highly branched and ramifying tubular epithelium has formed from the protodifferentiated epithelium (Fig. 7). Acini form at the tips of the branches, and islets form near the core of the epithelium, in close apposition to major ducts and associated blood vessels [159, 160]. In this section, we describe the developmental processes that occur during the secondary transition and the extrinsic and intrinsic factors that control these processes.

Pancreatic Bud Lobulation and Branching

Following the initial outgrowth of the early pancreatic epithelium (9–12 dpc), the bud transforms from a small, featureless mass of epithelium to a highly branched gland. As the gut tube undergoes "turning," a process that breaks bilateral symmetry of the alimentary tract and changes the positions of digestive organs relative to one another, the dorsal epithelium extends from an overall "fist-like" to a "bat-like" shape as it begins to extend numerous lateral (90° from the main axis) branches along its proximo-distal axis. Approximately 80% of branching events are lateral, the remaining bifid [161]. Small lobulations form along each lateral branch. Here, we define "lobulation" as the formation of multiple short blunt branches, or "lobules" [162, 163], while we refer to "branching" as the extension of longer, definitive epithelial branches with multiple lobulations and a predictable organization, which generate the main branches of the maturing organ [10]. While it remains unclear what geometrical parameters dictate cell movements that drive branch formation, it is believed that plexus formation, cellular proliferation, and epithelial remodeling are likely drivers of internal bud expansion, rather than simple extension of branch tips. These events set in motion the next phase of pancreatic development: the secondary transition. A better understanding of branching is likely to yield critical insights into pancreatic fate, as architectural changes of the epithelium have been associated with allocation of progenitors to the different pancreatic cell lineages [117, 118, 164].

The Secondary Transition

The secondary transition, as defined by Rutter and colleagues [8, 165], is the period of exponential increased accumulation of islet hormones and acinar digestive enzymes. By these criteria, this corresponds to the developmental window

encompassing the exhaustion of MPCs and the establishment of the bipotent tubular trunk/plexus with ductal and endocrine-restricted progenitor cells and the epithelial tips with committed, proliferation-competent acinar cell progenitors (Fig. 6). In the trunk/plexus domain, committed endocrine cells delaminate, cluster, and remain in close association with the epithelium. At the tips, committed acinar cells begin their final phase of differentiation.

The secondary transition initiates at about 12 dpc from multipotent precursor cells (MPCs) scattered throughout much of the epithelium including the periphery [69, 164, 166]. The MPCs were identified from a combination of several developmental markers with partially restricted expression: Ptf1a at a high level, Pdx1, Hnf1b, low carboxypeptidase A1 (Cpa1), and high c-Myc, consistent with high replication rate of these cells and the requirement for Myc to attain normal acinar cell mass [167]. MPCs also possess and require the TFs Sox9, Hes1, Mnx1, and Nr5a2 [58, 168, 169]. Importantly, other developmental markers are absent: Ngn3, endocrine hormones, Mist1, and acinar digestive enzymes other than Cpa1. Genetic lineage-tracing experiments of cells expressing *Cpa1* at 12.5 dpc showed that acinar, ductal, and islet cells all derive from the MPC population [164].

By about 14 dpc, the MPC population is exhausted [164, 166], converting to two compartments of replicating cells that characterize the secondary transition (Fig. 7). One within the tubular trunk regions produces precursor cells for islets and ducts and the other around the periphery for acinar cells. A high rate of cell proliferation propels the pro-acinar epithelial tips outward, while a slower rate of cell division expands the partly differentiated core epithelium [5]. Branching of epithelial tips is

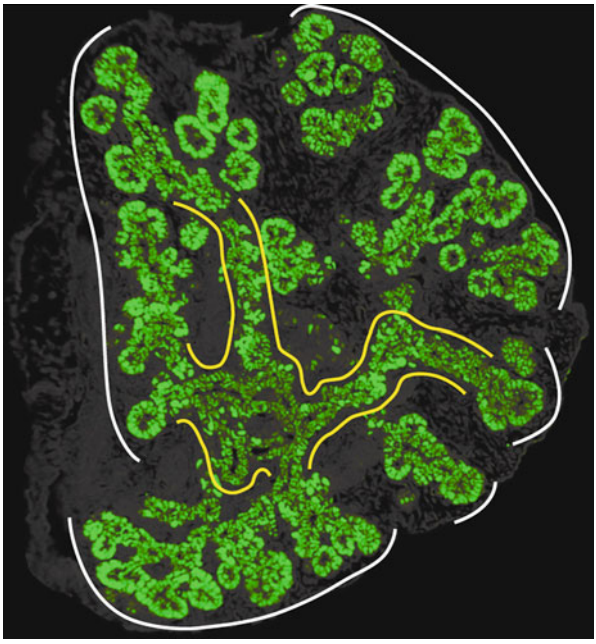

Fig. 7 The branched pancreatic epithelium during the secondary transition of an embryonic mouse pancreas. A section through the dorsal pancreas at late 14.5 dpc with immunolocalization of the transcription factor Pdx1 (*green*) displays the pancreatic epithelium during the secondary transition. At this stage, most of the cells of the epithelial tubules containing islet and ductal precursors (*yellow outlines*) and pro-acini (*white* indicators around the periphery) have nuclear Pdx1

in part driven by the formation of intervening "clefts" of differentiating tubule cells within clusters of tip cells [161, 164]. After 14 dpc, branch tips have committed to become acinar and begun the synthesis of the other secretory digestive enzymes, the core epithelium continues to generate endocrine cells from bipotent protodifferentiated epithelium.

Ductal and islet cells derive from the MPC progeny remaining in the trunk region of the epithelium. MPC daughter cells that enter this developmental compartment initially may be bipotent for the ductal and islet lineages [170]. Differing levels of Ngn3 distinguish three states of commitment: to endocrine development by high Ngn3, endocrine bias by low Ngn3, and ductal bias by the absence of Ngn3. Cells that then initiate expression of the TF Ngn3 to a high level initiate the islet developmental program [7, 124, 171, 172]. To date, an analogous transcriptional regulatory factor that commits precursor cells to the ductal lineage has not been identified. Ductal development may be the default option for the cells of the tubular epithelium that do not activate Ngn3 expression. Alternatively, bipotent MPC progeny may resolve quickly to more stable progenitors specified to either ductal or islet fate, which await further developmental cues. For the islet lineage, this is Notch signaling, which induces Ngn3 gene activation in a controlled temporal and spatial manner leading to the proper formation of committed endocrine cells that coalesce into islets [83].

In sum, the morphogenetic processes of the secondary transition generate a greatly expanded and branched tubular epithelium (Fig. 6) with regions specialized for the formation of islet cells near the center and acinar cell clusters toward the periphery. After this transition, the pancreatic epithelium has undergone three transformations: predifferentiated →1→ protodifferentiated →2→ tubular epithelium of ductal and endocrine progenitor cells with MPCs and differentiating acini at the tips →3→ differentiated ductal epithelium linking acini and separated from the delaminated endocrine cells. We consider next some of the developmental processes, both cellular and molecular, that create the acini, ducts, and islets.

Formation of Acini at Epithelial Tips

As the epithelial expansion of the secondary transition runs its course, MPCs commit to the acinar lineage, continue to replicate, and differentiate to pro-acinar cell clusters. To form acini, the pro-acinar cells at the ends of the precursor tubules may alter their cell-cell contacts and extend back over the tubule to form a cap of acinar cells (Fig. 8) [169]. This process is consistent with a developmental intermediate of a mature acinus with the terminus of the intercalated duct (aka centroacinar cells) inserting deep into the acinus [12].

The resolution of pro-acinar tip cells from the MPCs depends on both intrinsic and extrinsic factors, the interaction between which remains imperfectly understood (Figs. 3 and 4). One of the first signs of tip-trunk separation is the reorganization of Nkx6.1 and Ptf1a expression [173]. These TFs are initially present together in MPCs, but as MPCs generate bipotent trunk and acinar-committed tip cells, Nkx6.1 segregates to the trunk and Ptf1a to the tips (Fig. 8). In *Nkx6.1* mutant embryos at the MPC stage, Ptf1a is present ectopically in the trunk cells, and many

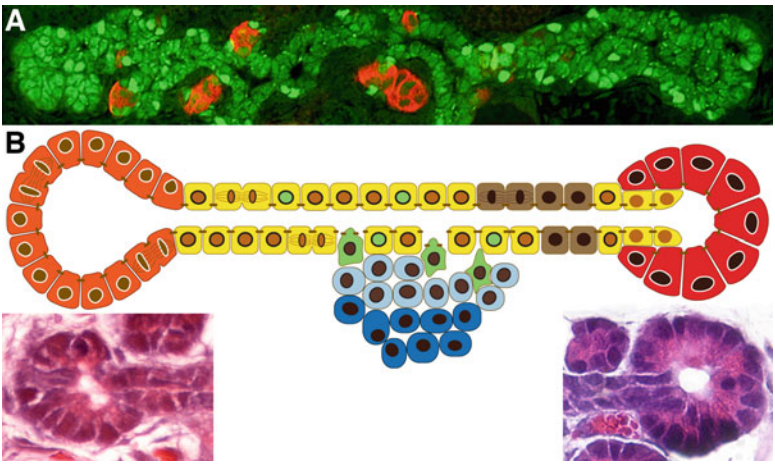

Fig. 8 A stereotypic model for the morphogenetic processes that generate islets centrally and acini peripherally. (**a**) A closeup view of the structure of Pdx1-expressing tubules, peritubular cords, and pro-acini from a section nearby that of Fig. 6. Note the subset of cells located in the cell cords or the tubules in contact with the cords that have very high Pdx1; these may be cells committed selectively to the β-cell differentiation program [270]. *Green*, Pdx1; *red*, glucagon, which marks the majority of the differentiated endocrine cells at this stage (14.5 dpc). (**b**) Diagram of the proposed developmental compartments of the post-MPC epithelium. The disposition of cell-cell junctions is not confirmed. *Yellow*, progenitors of islet and ductal cells retain the capacity for cell proliferation. *Beige*, committed ductal cell precursors. *Burnt orange* (*left*), committed pro-acinar cells retain cell proliferation and continuity with the tubular epithelium and have begun the synthesis of other digestive enzymes in addition to Cpa1. *Red* (*right*), differentiating acinar cells with low cell-replication capacity, ongoing cytodifferentiation, and accumulation of secretory (zymogen) granules. The acinar cells have formed a cap engulfing the tubule end cells, which become the centroacinar cells of the mature acinus. *Green*-to-*blue*, islet precursors initiate the islet program via *Ngn3* expression (*green* nuclei) and release from the tubule epithelium. Pre-endocrine cells in the epithelium break intracellular junctions, acquire transient mesenchymal properties, migrate from the epithelium, congregate in clusters, reestablish epithelial cell properties, and differentiate. *Inset left*: pro-acinus with connecting tubule. *Inset right*: differentiating acinus with cap structure

more acinar cells form. Conversely, forced continuous expression of *Nkx6.1* suppresses *Ptf1a* expression and acinar cell differentiation at this stage. In a complementary fashion, continuous expression of *Ptf1a* suppresses *Nkx6.1* and endocrine cell development. Thus, antagonism between these two fate-determining transcription factors leads to the resolution of the bipotent and acinar lineages through their segregated expression.

Among extrinsic signals, those mediated by β-catenin and the Notch pathway appear particularly critical for establishing the pro-acinar tip population (Fig. 4). Deletion of β-catenin produces an almost acinar-deficient pancreas, due to an accelerated depletion of MPCs into the bipotent duct-endocrine trunk fate at the expense of tip cells [48, 50, 174]. This phenotype correlates with downregulation of *Ptf1a* precisely at the onset of the secondary transition, indicating a key role for β-catenin in maintaining, but not establishing, the expression of this key acinar

determinant [50]. Polarized β-catenin activity, at the tips of the branching epithelium, could therefore serve as a "symmetry-breaking" process to tip the balance of the Ptf1a-Nkx6.1 antagonism described above.

Given that the mesenchyme into which the tip cells branch is known to have acinar-inducing activity, as described above, it is attractive to hypothesize that this tissue serves as a source of Wnt ligands, responsible for activating β-catenin/LEF-TCF-mediated transcription. Several observations argue against this, however. Transgenic reporters of Wnt/β-catenin/LEF-TCF activity exhibit little or no expression in the developing pancreas and no enrichment in MPCs or pro-acinar tips [175]. Pancreatic expression of a dominant-negative Frizzled 8 receptor, competent to block a wide variety of Wnt ligands, inhibits proliferation of progenitor cells but does not impair acinar development [176]. Impaired proliferation is observed in pancreas-specific β-catenin knockouts, as well as in *Wnt7b* knockouts described above, but this appears to be a separate phenomenon from the loss of pro-acinar tips [50, 51]. Given accumulating evidence that cell-cell adhesion cues play important roles in pancreas development, it is increasingly plausible that the functions of β-catenin in MPC patterning and acinar development are mediated by its signaling-independent functions downstream of E-cadherin [118, 119].

The role of β-catenin in acinar development appears to be approximately opposite to those of the Notch signaling pathway, and, indeed, the loss of acinar differentiation in the absence of β-catenin can be partially rescued by inhibition of Notch signaling [50]. In normal development, the expression of the Notch target gene *Hes1* in the precursor epithelium of the secondary transition extends up to, but does not include, the pro-acinar cells [148, 177], suggesting that Notch signaling may control this developmental boundary. Ectopic activation of Notch inhibits acinar development, in part by tipping the Ptf1a-Nkx6.1 balance in favor of the latter TF [173, 177, 178]. Conversely, development of pro-acinar tip cells is enhanced when Notch signaling is inhibited in MPCs, by deletion of *Hes1* or the Notch ligand co-factor *Mib1*, or expression of a dominant negative form of the Notch-Rbpj co-factor Mastermind-like [54, 179]. Thus, Notch signaling plays opposite roles, early and late, in determining acinar cell numbers: the pathway must be active in the primary transition to support progenitor cell expansion and then inactivated for segregation of pro-acinar cells (Fig. 4).

Pro-acinar cells derived from MPCs lose the transcriptional regulators that maintain the progenitor status of the epithelium. Hes1 is not detected in cells expressing amylase, and the TFs Sox9, Mnx1, and Hnf1b rapidly decline in these cells [148, 166, 169, 180]. However, *Ptf1a* expression continues at a high level in pro-acini, whereas it is shut off in the tubules containing the ductal and islet precursors. In this new context, Ptf1a acquires a new developmental function, which is to direct the differentiation of acinar cells. The active form of Ptf1a during early development is the trimeric PTF1-J complex (section "Initial Growth of Pancreatic Buds and the Primary Developmental Transition (9–12 dpc)"), which is necessary to initiate the formation of acini. Ptf1a, as part of the PTF1 complex, and Nr5a2 sit near the top of a transcription factor network that directs acinar development.

An early step in acinar differentiation is the synthesis of Rbpj-like (Rbpjl), the product of an *Rbpj* gene that was duplicated sometime during vertebrate evolution and since diverged. Whereas Rbpj is the transcriptional mediator for Notch, Rbpjl has lost the ability to participate in the Notch signaling pathway [181]. Rbpjl expression is largely limited to acinar cells of the pancreas and discrete regions of the forebrain. Transcription of the *Rbpjl* gene is activated in pro-acinar cells by the PTF1-J complex bound to the *Rbpjl* promoter [149]. As Rbpjl protein accumulates, it replaces the Rbpj subunit in the PTF1 complex. It is the Rbpjl form (PTF1-L) that binds and drives the promoters of most, if not all, the secretory digestive enzymes of differentiated acinar cells [82]. PTF1-L also replaces PTF1-J on the Rbpjl promoter and creates a positive regulatory loop that ensures the continued production of Rbpjl in acinar cells. In a complementary fashion, the Ptf1a gene has a transcriptional enhancer with a PTF1-binding site that requires the presence of a trimeric PTF1 complex for activity [182]. Consequently, the genes for both pancreas-restricted subunits of the complex are auto-activated in acinar cells by PTF1-L. Similar transcriptional positive feedback loops are commonly found near the top of a regulatory hierarchy in developing systems, and serve to first drive development toward a particular state, and then to stabilize that state [183]. It is likely that the PTF1-J complex helps establish the MPC population. The fact that *Rbpj* is required for acinar formation [141, 142] may be due to its role in the PTF1-J complex of the MPCs and not its role in Notch signaling, particularly as other components of the Notch pathway actively inhibit, rather than promote, acinar development. Ensuring the continued transcription of Ptf1a and Rbpjl through their autoregulatory loops drives acinar differentiation to completion.

In the adult, PTF1-L maintains the differentiated phenotype of pancreatic acinar cells. The complex resides on the enhancers or promoters of 34 of the 37 genes encoding the secretory digestive enzymes and is required for their continued transcription [82]. The loss of Ptf1a disrupts acinar cell identity and greatly increases susceptibility to KRAS-induced neoplasia [81].

Nr5a2/Lrh1, a member of the family of nuclear hormone receptors, is required during early embryonic development and subsequently for the formation of the pancreas during organogenesis. The lack of Nr5a2 leads to disruption of the primitive streak and failure of gastrulation [184]. During pancreatic development, it is required for the formation of the MPC population and a proper ductal tree, subsequently for allocation to the acinar lineage, and finally to complete acinar cell differentiation [169]. In the adult, Nr5a2 collaborates with the PTF1 complex to maintain genes for specialized acinar functions and likely controls much the same set of genes during acinar differentiation [185]. The induced loss of Nr5a2 from midgestation confers a heighted sensitivity to neoplastic transformation by oncogenic KRAS [186].

The bHLH TF Mist1 is present selectively in the serous-type secretory cells of many exocrine glands [187] and helps establish high-capacity secretory phenotype of those cells [188]. During pancreatic development, Mist1 is required to establish proper apical-basal cell polarity and complete acinar differentiation [189]. Mist1 acts downstream of Ptf1a, because Mist1-deficient embryos initiate acinar development

normally, but the acinar cells do not acquire proper cytoarchitecture or regulated exocytosis. Indeed, PTF1-L binds the pancreatic transcriptional enhancer of *Mist1* and drives *Mist1* transcription [190]. In the absence of Mist1, acinar cells lose intercellular communication because gap junctions do not form properly [191], have mitochondria with compromised Ca++ uptake and Golgi positioned incorrectly [192], and have defective regulated exocytosis [193]. As a consequence of these defects in gene expression and cellular organization, normal acinar cell polarity is not established, Ca++ signaling is abnormal, packaging the secretory enzymes is defective, intracellular zymogens are activated, and genes characteristic of duct cells are expressed aberrantly [191–193]. Mist1 assists acinar differentiation independently and in collaboration with the PTF1-L complex. In adult acini, Mist1 and PTF1-L together bind and regulate more than 100 downstream genes for specialized acinar cell functions such as secretory protein synthesis and processing, exocytosis, and robust maintenance of endoplasmic reticulum homeostasis [190]. Mist1 also collaborates with Xbp1 to maintain a vigorous unfolded protein response system [194], a critical aspect of the acinar phenotype. Mist1 also limits acinar cell replication by controlling the expression of the cell cycle regulator p21 [195]. Thus, Mist1 controls the final stage of differentiation that establishes the functional and stable acinar cell phenotype.

The zinc-finger TFs Gata4 and Gata6 are present throughout the early pancreatic epithelium [196]. Gata4 becomes restricted in the tips of epithelial branches during the secondary transition and is present exclusively in the acinar cells of the mature gland [196, 197]. In contrast, Gata6 segregates to the ducts and their associated endocrine cell cords. Function follows distribution: at this developmental stage, Gata4 is needed for the proper number and maturation of acinar cells and Gata6 for ducts [153, 198].

The HD protein Prox1 is required for the proper allocation of progenitor cells to the endocrine versus exocrine lineage. *Prox1*-deficient embryos have precocious acinar development and diminished total acinar and islet tissue formation [192]. These developmental defects suggest that Prox1 might help maintain the multipotent progenitor cell population by delaying acinar development. Because Prox1 can interact with and inhibit the transcriptional activity of Nr5a2 in other contexts [199], it might govern the orderly formation of acini by restraining Nr5a2 function during pancreatic development. Indeed, Prox1 and Nr5a2 are expressed in complementary patterns during the secondary transition. Just as eliminating the restraining effects of Notch signaling on Ngn3 activity causes progenitor cell depletion by allowing precocious endocrine development, so too might the absence of Prox1 allow unrestrained Nr5a2 activity and the premature induction of acinar development. Prox1 deficiency retards the early growth of the embryonic pancreas. Before birth, growth restores normal amounts of acinar and islet tissue, and inter- and intralobular ducts acquire larger than normal diameters due to greater numbers of cells [200]. Ductal developmental regulators Sox9, Hnf6, and Hnf1b were unaffected, so that Prox1 appears to affect principally growth, although functional studies of mature ducts were not reported.

Ductal Development

The ductal system of the mature pancreas comprises the two main pancreatic ducts that drain into the intestine, small interlobular ducts that link the lobules to the main drainage, smaller intralobular ducts, and even finer intercalating ducts (IDs) that connect to individual acini [201, 202]. In addition, the pancreas is the only exocrine gland in which the connecting ducts (here intercalated ducts) insert into the acinus. These extensions of the intercalated duct have been designated "centroacinar cells," a term that obscures their function, origin, and relationship with the ductal tree, and we suggest instead the designation intra-acinar duct cells (IAD cells). The ductal nature of the IAD cells is indicated by their expression of the TF Sox9, the intermediate filament cytokeratin-19 (CK19), and the transmembrane protein CD133, all of which becomes duct restricted after the secondary transition [168, 203, 204].

The evidence that the IDs and IADs derive from a developmental program distinct from that of large ducts is severalfold. The two programs can be resolved by the gestational times at which each requires Pdx1 [77]: depleting Pdx1 experimentally just prior to the secondary transition at 12.5 dpc allows the formation of the large ducts, but not IDs or IAD cells. The ductal structure that forms upon Pdx1 depletion at sequential time-points appears to represent incomplete main ducts (one from each bud), primary branches from the main ducts (the interlobular ducts), and the beginning of secondary branches distally (intralobular ducts). In a similar fashion, the directed germline inactivation of *Ptf1a* leads to the formation of the large but not the small ducts [74, 75, 149], while the opposite phenotype is observed in *Hnf6* mutant pancreata [205]. The Notch signaling pathway appears to be a critical determinant of duct cell development: whereas activation of this pathway prior to the secondary transition induces a trunk progenitor-like fate, its activation after the secondary transition induces mature duct cell differentiation [58]. Conversely, targeted disruption of the Notch ligands *Jagged1* and *Jagged2* results in failure of IAD development in later embryogenesis, while deletion of the Notch partner TF *Rbpj* in adult IADs causes their re-specification into acinar cells [203, 204]. The increasing availability of tools to mark and manipulate different classes of duct cells is likely to provide new insights into pancreatic cancer as well, such as the recent appreciation that duct cells give rise to IPMN precursor lesions rather than PanINs [206, 207].

Resolution of the Epithelial Plexus

The transformation of the epithelial plexus into the ramifying ductal network of the exocrine pancreas, with its interspersed islets, has only recently been elucidated. Previously thought to develop by the more conventional mechanisms of bud tip extension and branching, as in the lung, the pancreas is now understood to arise via formation and resolution of a complex plexus [10, 117]. Transiently 3D, the plexus undergoes remodeling and resolution, as rungs of the plexus ladder either regress or enlarge, to yield a tree-like network. However, it remains unclear how this occurs at the cellular level, and the molecular underpinnings of these processes remain unknown. Understanding this process has become of particular interest, since a

recent report has identified this early epithelial plexus as the niche for endocrine progenitors [11]. Indeed, $Sox9^+/Ngn3^+$ progenitors are found primarily within this region, and EdU pulse-chase experiments show that the secondary transition occurs at the height of plexus remodeling. Furthermore, during this burst of endocrine differentiation, progenitors take about 12 h to transit through to delamination and differentiation, as Notch signaling functions to maintain the pool of progenitors. Upon full resolution of the plexus at perinatal stages, ducts and finer branches fully emerge, and progenitors become exhausted. These findings identify the epithelial plexus as the niche for endocrine differentiation in the embryonic pancreas; however, it underscores that our understanding of cell-cell relationships within this microenvironment is still in its infancy.

A number of TFs are critical to formation of the ductal network. Sox9 is one such TF, which is expressed throughout the early epithelial plexus, which is required for ductal as well as endocrine and exocrine lineages [69]. The early epithelium expresses Sox9 widely and secondary transition trunk epithelium contains $Sox9^+$ bipotent ducto-endocrine progenitor cells [69, 168]. During plexus resolution, cells asynchronously flatten and lose Sox9 expression as they acquire ductal fate [208]. Loss of Sox9 results in a cystic pancreas with scattered acini and near total loss of endocrine cells [85]. In addition to being required for endocrine specification, it is also known to be required to maintain pancreatic ductal identity. In the adult pancreas, it is restricted to duct and centroacinar cells and is required to maintain ductal integrity and primary cilia formation [83, 209].

Similarly, Hnf6 and the TFs it controls are critical regulators of ductal development. The absence of Hnf6 causes extensive developmental defects of the pancreas [210]. Whereas the extent and morphology of acinar tissue is near normal and the first-wave endocrine cells form, the second-wave lineage does not appear, and dilated cystic duct structures appear in the epithelium. The cystic ductal phenotype appears at 15 dpc, which may mark the onset of duct-specific differentiation. The cystic ducts express the differentiation marker Muc1 but are devoid of the primary cilia normally present throughout the mature ductal tree [205]. Mutations in the genes for the structural proteins of the cilium cause similar defects in ductal differentiation, but the second-wave endocrine cells form nonetheless [211]. These observations suggest that ductal precursors form in Hnf6-deficient pancreas but do not differentiate properly, in part due to the absence of primary cilia.

Hnf6 controls ductal morphogenesis including ciliogenesis via two additional TFs, Hnf1b, and Glis3. Hnf6 is needed for the expression of Hnf1b during liver and pancreatic development [212], and Hnf1b is known to control the expression of genes for cilium function in the kidney [213]. The absence of Hnf1b in the cells of the cystic ducts of Hnf6-deficient embryonic pancreas [205, 211] indicates that the cilium defect is due directly to the loss of Hnf1b. In turn, Hnf1b binds and controls the transcription of *Glis3*, a Zn-finger TF also necessary for ductal cilia. Glis3 first appears in the bipotent trunk progenitor cells, segregates to ductal, β- and PP cells [214], and functions in each of these three developmental compartments [215]. The pancreas of embryos bearing a functionally impaired *Glis3* forms cystic ducts [215] due to disrupted primary cilia.

Notch signaling is necessary for proper formation of ducts through the induction and maintenance of Sox9 and Hnf1b expression [83, 216]. In this context, Sox9 induces genes directing ductal differentiation and cilia that complement the set of genes controlled by Hnf1b [83]. The level of Notch signal received by bipotent trunk cells affects their decision to remain on the path to duct development or veer off to the islet cell fate (see below).

The Second Wave of Endocrine Cells: Formation of Primitive Islets

A new population of endocrine cells, distinct from the first wave of the early pancreatic bud, arises during the secondary transition from a population of progenitors left behind by the advancing epithelial tips (see Fig. 7). Several excellent reviews describe islet cell specification and development comprehensively [5, 25, 217].

A transient, intense expression of the *Ngn3* in scattered cells of the tubular plexus epithelium commits these cells to cease proliferation [17, 218] and begin the islet developmental program [56, 178, 219]. Notch signaling within the specialized environment of the mid-development epithelial plexus described earlier nurtures pre-endocrine progenitors by sustaining proliferative growth and suppressing differentiation [11]. A recent study indicates that the "bipotent trunk" region has a subpopulation of cells that are biased toward the endocrine fate with *Ngn3* transcription, but low-Ngn3 protein [170]. Two likely fates for those low-Ngn3 cells are proposed: replication to maintain the endocrine-biased progenitor population and derepression of *Ngn3* to high functional levels to initiate endocrine cell development. Studies on *Ngn3*-knockout mice showed without the generation of endocrine cells, transcription of the Ngn3 locus was increased [220], suggesting that differentiating endocrine cells may produce Notch ligands that normally control the balance between the low (suppressed) Ngn3 progenitors and the high (derepressed) Ngn3 precursors specified to endocrine development [11].

The activation of *Ngn3* is promoted directly by the binding of Hnf1b, Hnf6, Glis3, Pdx1, Foxa2, and Sox9 to distal regulatory regions of the *Ngn3* gene [210, 221–224]. These TFs are present throughout the interior region of the precursor epithelium, but high *Ngn3* expression is repressed in all but a few scattered cells at any one instant by Notch signaling [221]. Strong localized Notch signaling favors progenitor status and duct cell formation by maintaining high levels of Hes1, which binds and represses the *Ngn3* promoter [177, 178]. Lower Notch signaling is unable to maintain effective Hes1 levels but still drives transcription of *Sox9*, which in turn drives *Ngn3* transcription, and the balance is tipped toward endocrine development [86]. Limited *Ngn3* expression provides a measured induction of endocrine development without exhausting the progenitor population prematurely and without preempting ductal cell development from the same population. By the time islet precursors become committed and leave the epithelial tubule, *Ngn3* transcription is abruptly shut off by feedback repression of *Sox9* by Ngn3 [86].

Ngn3 initiates a developmental cascade by activating the promoters of genes for TFs with roles in endocrine differentiation (Fig. 3: "islet precursor" set) [217]. Neurod1, Insm1, and Rfx6 are downstream and likely direct targets of Ngn3 [225]. Rfx6 helps establish the common islet lineage [226, 227] and is required

subsequently to maintain the functional identity of β-cells [228]. Insm1 facilitates endocrine cell differentiation by suppressing the endocrine-progenitor program and inducing genes necessary for proper islet cell differentiation [229]. The action of Neurod1 favors the formation of β-cells over α-cells [230].

Genes encoding intrinsic factors that resolve the α, β, δ, ε, and PP sub-lineages of islet cells are among the set of endocrine regulatory genes induced by Ngn3. Transcription factors critical to specifying individual islet cell lineages and their final differentiation include Pdx1, Foxa2, Neurod1, Pax4, Arx, Rfx6, Nkx6.1, Mnx1, Insm1, Glis3, Isl1, Nkx2.2, Pax6, and MafA. More complete descriptions of the TFs that control islet cell differentiation are presented in several excellent reviews [104, 180, 231–233].

Extrinsic factors also regulate the second wave of endocrine differentiation. The control of the commitment to endocrine fate by Notch signaling has been well characterized by experimental manipulation. Driving Notch1-ICD expression in precursors to both endocrine and exocrine lineages prevents the differentiation of both compartments and leaves an incompletely differentiated ductal epithelium [178]. The in vivo overexpression of constitutively active Notch (Notch1-ICD) selectively in the Ngn3+ precursor population also suppresses endocrine differentiation [234].

Formation of Islet Precursor Cells by Delamination

Whereas acini form at the ends of precursor tubules and maintain topological continuity of with the ductal system, islets form from cells that escape the continuum of the epithelial tubules (Fig. 8). The escaped islet precursor cells coalesce into cords that remain intimately associated with and within the basal lamina of the single-cell layer tubules [8]. The endocrine cell cords grow by continued recruitment of precursors from the epithelial tubules, rather than by replication of the differentiating endocrine cells.

The endocrine cell cords are thus endocrine cells early in their differentiation process and can be distinguished from the tubules by the presence of synaptophysin, a component of the microvesicle secretory machinery and an early differentiation marker of neuroendocrine cells [16]. Cord cells with synaptophysin, but without any of the five principal islet hormones, appear to constitute the less differentiated cells most recently released from the tubules. Approximately half of the synaptophysin-expressing cells at 14.5–15.5 dpc are pre-hormone precursors, while the remainders have endocrine hormones and are therefore more differentiated. As the endocrine cords mature, increase in size, and form spherical structures, the basal lamina surrounding the forming islet eventually pinches off near its association with the differentiating duct and thereby separates the extracellular spaces of the endocrine and exocrine tissues.

Cells committed to the islet lineages are released from the pancreatic epithelium by a version of the developmental epithelial-to-mesenchymal transition (EMT) [14]. During EMT, epithelial cells escape their epithelial neighbors by dismantling tight junctions and acquiring modest mesenchymal cell properties [235]. For this process to be a viable option for islet cell derivation, reversion from a transient mesenchymal state back to an epithelial state must occur prior to endocrine differentiation within the endocrine cell cords. The evidence for EMT during the

endocrine development of the secondary transition is threefold: lower levels of E-Cadherin and increased Vimentin in delaminating cells [14] and the appearance of Snail2 (a known inducer of EMT in other contexts) in scattered cells of the tubular precursor epithelium at the appropriate time for initiating EMT shortly after precursor cells commit to endocrine fate [236].

Comparison of First and Secondary Waves of Endocrine Cells

The precise developmental relationship between the first- and second-wave endocrine cells is unknown; however, differences between them suggest that they represent different cellular lineages [237].

- Although both first- and second-wave endocrine cells require *Ngn3* [238], the formation of the first-wave cells does not require *Pdx1* [72, 146] or *Ptf1a* [74], which are critical to the formation of the second-wave lineage. Indeed, only a few of the first wave cells express *Pdx1*.
- Many more β-cells than α-cells are made during the secondary transition. In addition, glucagon and insulin co-expressing endocrine cells are not observed following the secondary transition. The ratio of β- and α-cells seems to be an inherent property of the two lineages, because experimental manipulation by superinduction of Ngn3 to high levels during the primary transition leads to overproduction of glucagon-cells and during the secondary transition leads to overproduction of β-cells [124].
- Clusters of first-wave endocrine cells are invariably connected to the precursor epithelium by a cellular bridge and appear to separate from the protodifferentiated endoderm by a budding process [239], rather than the delamination that occurs during the secondary transition.
- Whereas the α-cells that form during the secondary transition use prohormone convertase 2 (PC2) to process the proglucagon polypeptide precursor to active glucagon, the early cells have PC1/PC3 rather than PC2 and cleave the precursor to GLP1 and GLP2 [240]. Because the glucagon-expressing first-wave cells have PC1/PC3 and produce the GLP peptides, they are not strictly α-cells. If these cells contribute to the α-cell population of neonatal islets, as proposed, they must switch to PC2 from PC1/PC3 to produce glucagon. Processing proglucagon to GLP1 and GLP2 by PC1/PC3 is a characteristic of the enteroendocrine L cells of the intestine and stomach. Thus, the early endocrine cells may be closely related to an enteroendocrine lineage, which also requires Ngn3.

The developmental origins and fates of the first- and second-wave cells are notable in two respects. *First*, the cells of the first wave are not the progenitors of the second [7, 125]. Consequently, two separate endocrine programs occur rather than a single, continuous one. *Second*, an equivalent, predominately glucagon-expressing, first-wave endocrine cell population does not occur during human pancreatic development [4, 127]. Instead, insulin cells appear first and are always prevalent, followed shortly by the appearance of glucagon and somatostatin cells. These earliest human endocrine cells form during a period of morphogenesis that appears related to the murine second

transition, rather than an earlier primary transition. For comparisons with the developmental processes of human islet formation, it is important to distinguish the first and second waves of murine endocrine cells.

Dorsal and Ventral Bud Fusion

As the dorsal and ventral buds grow, branch, and extend, they are brought into contact at the base of their primary ducts by the movements of gut turning. The primary ducts fuse at 11.5 dpc, while their distal portions remain largely separate. In humans, fusion of the dorsal and ventral buds (~35 days) creates a more integrated organ than in rodents. The dorsal bud forms the upper part of the head of the human pancreas, as well as the main body and tail (or splenic portion). The ventral bud forms the lower part of the head of pancreas – the uncinate process. The compositions of the dorsal and ventral portions differ. The dorsal pancreas forms more abundant large islets, with a higher number of β- and α-cells and a smaller number of PP cells. In contrast, the ventral pancreas is interspersed with smaller islets that contain proportionally more PP cells [241]. However, the relative numbers of islets within the two sections of the pancreas are comparable [242].

Perinatal Growth and Differentiation (16 dpc to Neonate)

Following the secondary transition and the acquisition of acinar, ductal, or endocrine cell fates, the pancreas continues growth in parallel with most other embryonic organs. The pancreas expands by cell proliferation with exocrine tissue added at the periphery and endocrine cells coalescing into progressively larger and more mature clusters. The proportion of endocrine cells declines due to the massive expansion of maturing exocrine tissue. During the first few weeks after birth, the first mature islets become distinguishable with the recognizable architecture of a β-cell core surrounded by a mantle of alpha, epsilon, and recently emerged delta and PP cells (which begin to appear at 15.5 dpc).

Isletogenesis

Islet morphogenesis begins at the secondary transition with the endocrine cell precursors released from the tubular pancreatic epithelium. Unlike the first-wave cells, these pre-endocrine cells aggregate into ribbon-like cords that remain in close association with the precursor epithelium. The cells migrate along rather than away from the underlying epithelium, and not far from their origin. Shortly before birth, glucagon-expressing α-cells begin to envelop the β-cell cords [239], initiating the formation of a peripheral mantle in mature islets. The cords of mixed endocrine cells have been proposed to be broken up by the growth of acinar tissue, which intercedes and divides the cords into segments, like beads-on-a-string [7]. Shortly before birth, the forming islets acquire a characteristic spherical

shape, lose their tight association with the ductal epithelium, and organize nearby within the acinar parenchyma [8]. An important aspect of islet morphogenesis is the internal organization of β-cells into tightly bundled and polarized epithelial sheaths, perfused by numerous fine capillaries [243]. The polarity of β-cells around individual blood vessels is dependent on the serine threonine kinase Lkb1 [244]. Similar to mouse, human islets arise primarily in the core region of the pancreatic bud [245] but have a markedly different mature organization, with α-, β-, and δ-cells distributed throughout the islets [246]. Although isletogenesis is readily observed in the developing pancreas, and the term widely used, it has been almost completely ignored by researchers, with only a few exceptions outlined below.

Mutations in a number of key developmental extrinsic and intrinsic factors disrupt the morphogenesis of normal islets. The defects fall into one of two main categories: disruption of intra-islet organization or aberrant islet growth, which have been observed in mouse models of diabetes [247–250]. Loss of function of an intrinsic factor, the GTPase Rac1, leads to impaired migration of endocrine progenitors following delamination, which causes aberrant increase in cadherin-mediated cell-cell adhesion and retention of islets near ducts [251]. Disruption of BMP signaling via deletion of the BMP receptor 1a gene, for instance, disrupts the segregated distribution of α-cells to the mantle and β-cells to the interior and impairs glucose-stimulated insulin secretion [252]. Proper control of the matrix metalloproteinase MMP-2 by TGF-β1 also is required for normal islet morphogenesis [132]. Persistent expression of HNF6 beyond 18.5 dpc causes failure of islet architecture and β-cell dysfunction [253]. When Nkx2.2 is experimentally converted into a repressor (via fusion with the engrailed repressor domain) and expressed in the perinatal endocrine compartment, α-cells form within the islet core, and the affected mice become overtly diabetic after birth [99]. These are only a few of many similar examples of mutations that cause aberrant islet anatomy.

It is likely that much of the control of islet architecture by EF and TF pathways occurs via their regulation of cell-surface adhesion molecules or components of the extracellular matrix, which direct many aspects of tissue morphogenesis. Indeed, integrins and cell adhesion molecules, such as E-cadherin and NCAM, are downstream targets of TF and EF signaling pathways driving pancreatic development, and they have been directly implicated in guiding the migration and organization of endocrine cells into islets.

Conclusion

A complex and dynamic interplay of extrinsic and intrinsic signaling pathways create the cell diversity, anatomy, and finely tuned physiologic functions of the adult pancreas. Because each signaling pathway is used broadly during embryogenesis, pathway defects often cause early embryonic lethality, prior to the onset of

pancreatic organogenesis, and consequently pancreatic defects generally cannot be distinguished. In contrast, because most of the key pancreatic TFs discussed in this review have functions largely restricted to pancreatic development or function, many are directly linked to heritable human pancreatic maladies, including endocrine cell defects in diabetes [254] and exocrine agenesis [73, 76].

Defects in signaling pathways are common in human pancreatic cancers. Aberrations in Notch, TGFβ, Hedgehog, and Wnt pathways occur in adenocarcinoma [255–257] and are discussed in other chapters of this handbook. By contrast, mutations in genes encoding key pancreatic TFs that control acinar development are not commonly associated with human pancreatic cancer. The association of *Nr5a2* with pancreatic adenocarcinoma through a GWAS study [258] is the notable exception. Recent evidence from mouse genetic models of pancreatic adenocarcinoma strongly links acinar cell dedifferentiation with susceptibility to transformation by oncogenic KRAS. [34, 259]. Inactivation of *Mist1*, *Nr5a2*, *Gata6,* or *Ptf1a* leads to acinar dedifferentiation and the acquisition of non-acinar cell characteristics and enhances the pace and extent of transformation by oncogenic KRAS [81, 186, 260–262].

Understanding the complex relationships between these factors and how they influence pancreatic cell growth, proliferation and/or differentiation, will be critical to developing therapeutic approaches to diseases affecting a wide range of conditions from metabolic defects to pancreatic cancer (Box 5). One striking example is the recent demonstration that insulin gene expression can be induced in vivo by directed transdifferentiation of adult acinar cells through the forced expression of just three endocrine transcription factors, Pdx1, Ngn3, and MafA [263, 264], by expression of Pax4 [265] or by reduction of Ptf1a activity [266]. Refinement of this process may lead to a therapeutic approach to replace lost β-cell function in diabetics (Box 6). It is imaginable that similar approaches may someday provide the option of inducing acinar function to reverse exocrine pancreatic insufficiency.

Box 5 Future Directions

- Identify signals from vasculature and mesoderm that control pancreatic growth and differentiation.
- Define the cellular and molecular processes that underlie the formation of islet cell precursors by the delamination of cells from the pancreatic epithelium.
- Understand molecular and cellular consequences of defects in the extrinsic signaling pathways that control pancreatic organogenesis.
- Define the plasticity of exocrine and endocrine cell phenotypes that allow transdifferentiation.
- Delineate the intrinsic and extrinsic factors that maintain acinar cell identity.

Box 6 Clinical Implications

- An understanding of developmental factors involved in growth and differentiation lays the foundation for developing clinically relevant therapies for pancreatic exocrine cancer.
- Potential to translate an understanding of the formation and maintenance of acinar cell identity to mechanisms that resist neoplastic transformation. An emerging understanding of the development of ducts and acini may inform the design of treatments to restore exocrine tissue destroyed by disease.
- Understanding the key developmental factors has already led to the in vitro generation of beta cells for potential replacement therapy for diabetics.

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Cross-References

- ▶ Animal Modeling of Pancreatitis-to-Cancer Progression
- ▶ Epigenetics and Its Applications to the Progression Model of Pancreatic Cancer
- ▶ Hedgehog Signaling Plays a Dual Role in Pancreatic Carcinogenesis
- ▶ Mouse Models of Pancreatic Exocrine Cancer
- ▶ Notch Signaling in Pancreatic Morphogenesis and Pancreatic Cancer Pathogenesis
- ▶ Smad4-TGF-β Signaling Pathways in Pancreatic Cancer Pathogenesis

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The Molecular Pathology of Precursor Lesions of Pancreatic Cancer

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Abstract

It has become evident over the past decade that pancreatic ductal adenocarcinoma (PDAC) does not originate de novo, but rather, through a multistep progression that involves histologically defined precursor lesions. Three major subtypes of precursor lesions of PDAC have been identified to date, including pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasm (IPMN), and mucinous cystic neoplasm (MCN). PanINs constitute by far the most common precursor lesion, and are, by definition, microscopic in nature, while IPMNs and MCNs occur less frequently and are macroscopic (e.g., radiographically detectable) precursor lesions. In addition to the development of consensus histopathological criteria for the identification and classification of PDAC precursors, there has also been considerable progress made in characterizing the genetic alterations underlying these lesions. Elucidating the molecular pathology of precursor lesions has enabled a better understanding of the pathogenesis of early pancreatic neoplasia, and provided a seedbed for developing tools for early detection and chemoprevention of PDAC. The histopathology, molecular genetics as well as clinical implications and possible directions for future research of PanINs, IPMNs, and MCNs will be discussed in this chapter.

Keywords

Pancreatic ductal adenocarcinoma · Precursor neoplasms · Molecular genetics · Early detection · Pathogenesis

Introduction

The first example linking the progression from a noninvasive precursor lesion to invasive cancer with a cumulative sequence of genetic aberrations was established for the adenoma-carcinoma sequence in colon cancer [1]. This concept has since been extrapolated to many solid cancers, including pancreatic cancer or pancreatic ductal adenocarcinoma (PDAC). In fact, there is now increasing evidence to suggest, that almost all of the major epithelial malignancies may be associated with discrete noninvasive precursor lesions, and that histological progression of such lesions is paralleled by an underlying genetic progression. The general concept that PDAC does not arise de novo, but rather originates from tangible noninvasive precursor lesions, was first proposed over a century ago [2]. However, only over the past few decades have the identity of these precursor lesions been solidified through

Table 1 Clinicopathologic features of PanINs, IPMNs, and MCNs

	PanIN	IPMN	MCN
Predominant age	Prevalence increases with age	60–70 years	40–50 years
Female: male ratio	1:1	2:3	20:1
Preferential location	Head > body/tail	Head (80%) > body/tail	Body/tail (90%) > head
Ductal communication	N/A	Yes	No
Cyst contents	N/A	Viscous	Viscous
Stroma	Collagen-rich	Collagen rich	Ovarian type
Multifocal disease	Often	In ~20–30%	Extremely rare
EUS findings	Normal	Ampullary mucin extrusion, dilated pancreatic duct, and filling defects	None
Key genes involved in pathogenesis and progression	*KRAS*, *CDKN2A*, *TP53*, and *SMAD4*	*KRAS*, *GNAS*, *RNF43*, *CDKN2A*, *TP53*, *PIK3CA, PTEN*, and *SMAD4*	*KRAS*, *RNF43*, *CDKN2A*, *TP53*, *PIK3CA, PTEN*, and *SMAD4*

Abbreviations: *IPMN*, intraductal papillary mucinous neoplasm; *MCN*, mucinous cystic neoplasm; *N/A*, not applicable; *PanIN*, pancreatic intraepithelial neoplasia

meticulous histopathological and molecular biological analysis, and through introduction of a consensus nomenclature [3, 4]. Three different types of precursor lesions to PDAC are recognized: pancreatic intraepithelial neoplasia (PanIN), by far the most common, intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs). The key features of these three precursors are listed in Table 1, and each will be discussed independently within the text.

Pancreatic Intraepithelial Neoplasia (PanIN)

Clinical and Histopathological Features of PanINs

PanIN lesions are microscopic noninvasive precursor lesions with varying degrees of architectural and cytologic atypia, and are located in the interlobular ducts of <5 mm in diameter [3]. Based on the degree of both architectural and cytologic atypia, PanINs are divided into two grades: low-grade and high-grade. Low-grade PanINs consist of flat-to-papillary ductal epithelium with abundant supranuclear mucin. The nuclei may be round or elongated and basally oriented or show some loss of polarity, crowding, enlargement, pseudostratification, and hyperchromasia (Fig. 1a). Mitoses are only rarely seen, and when present, are basal and morphologically normal. In contrast, high-grade PanINs are characterized by significant architectural and cytologic atypia. These lesions are usually papillary and, in some instances, demonstrate

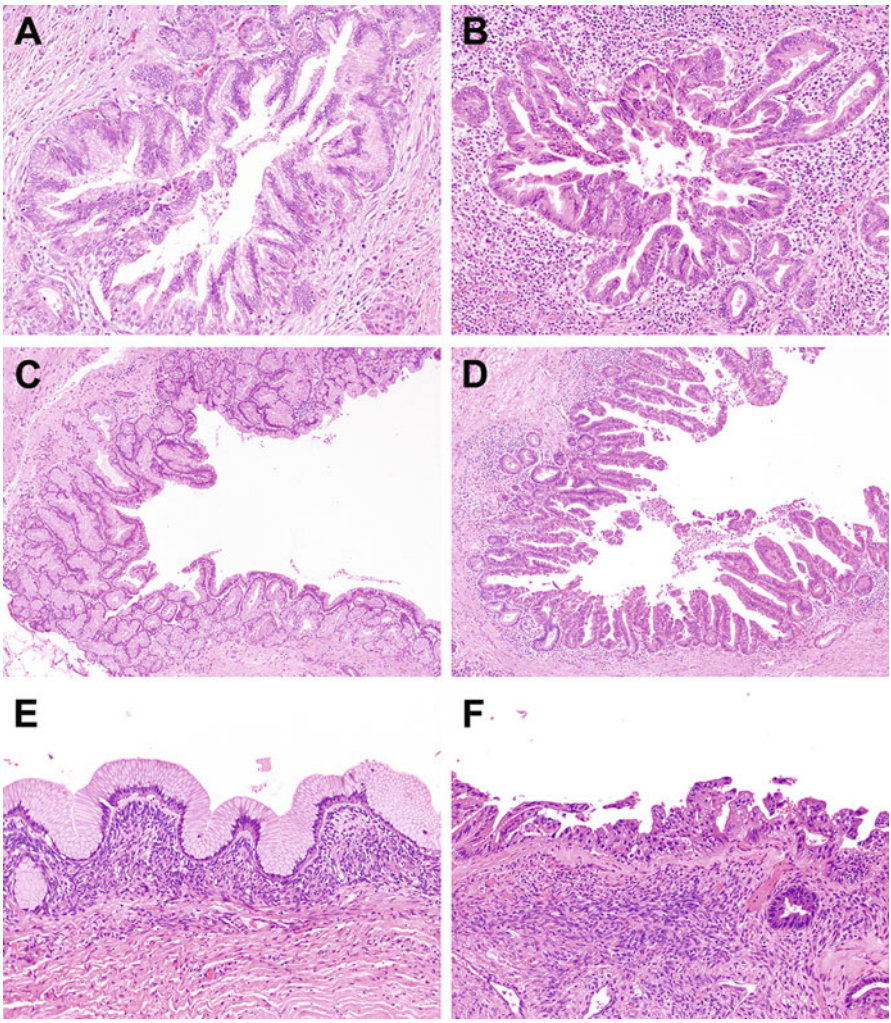

Fig. 1 Representative histologic sections of low-grade (**a**) and high-grade (**b**) PanINs, low-grade (**c**) and high-grade (**d**) IPMNs, and low-grade (**e**) and high-grade (**f**) MCNs. Note the presence of ovarian-type stroma underlying the mucinous epithelium of MCNs (**e**, **f**)

cribriform architecture and luminal necrosis. The nuclei are enlarged, hyperchromatic and show loss of orientation, such that they are no longer perpendicular to the basement membrane (Fig. 1b). Further, the nuclear-to-cytoplasmic ratio is significantly increased. Nucleoli may be prominent, and mitoses, some of which are luminal and atypical, may be present.

The overall prevalence of PanINs increases with age, and low-grade PanINs are found in over half of the population above the age of 65 years [5]. An increased

prevalence of PanINs in not only observed in PDAC, but also in the setting of chronic pancreatitis [6]. In one series, Andea and colleagues found PanIN lesions in 67 of 82 (82%) pancreata from patients with PDAC and in 54 of 86 (63%) of patients with chronic pancreatitis, but only in 10 of 36 (28%) patients with otherwise normal pancreata. Interestingly, PanINs are also frequently found adjacent to other periampullary neoplasms, including ampullary adenomas and adenocarcinomas, acinar cell carcinomas, well-differentiated pancreatic neuroendocrine tumors, serous cystadenomas, and solid-pseudopapillary neoplasms [7, 8].

Molecular Genetics of PanINs

The histological progression of PanIN lesions has been linked to progressive accumulation of genetic aberrations that are shared with PDAC. These aberrations do not occur in a random manner, but rather in a well-described sequence of early and later events (Fig. 2), as depicted in the PanIN progression model ("PanINgram").

Oncogene Mutations in PanIN Lesions

A growing number of oncogenes have been identified that contribute to pancreatic carcinogenesis upon activation, usually through intragenic mutations or copy number alterations. The most commonly observed activating point mutations in PDAC, as well as in PanINs, are found in the *KRAS* oncogene on chromosome 12p. These mutations, which are also among the earliest genetic alterations observed during pancreatic carcinogenesis, can be detected in up to 90% of PDACs and most often occur on codons 12, 13, or 61 [10, 11]. Utilizing pyrosequencing, a highly sensitive DNA sequencing technique, Kanda et al. showed more than 90% of low-grade PanINs harbor *KRAS* mutations, suggesting that this oncogene plays a critical role in PDAC initiation [12]. The importance of constitutively activated *KRAS* in PDAC initiation is further underscored by the development of genetically engineered mouse models of PDAC, wherein a mutant *Kras* allele is sufficient for the development of murine PanIN (mPanIN) lesions [13, 14]. Activating mutations impair the intrinsic GTPase activity of the *KRAS* gene product, leading to constitutive activation of downstream intracellular signaling cascades [15]. Three major downstream Ras effector cascades have been identified that are involved in mediating the oncogenic properties conferred by constitutively active *KRAS*, namely the RAF/MEK/ERK, the PI3K/AKT, and the RalGDS/Ral pathways. Of note, oncogenic Ras signaling seems to be involved in not only PDAC initiation, but also required for tumor maintenance in established cancers [16, 17]. Interestingly, in a proportion of PDAC more than one distinct mutation within the *KRAS* gene can be detected, suggesting that within the same organ, multifocal precursor lesions can develop independently from the one that eventually culminates in PDAC [18].

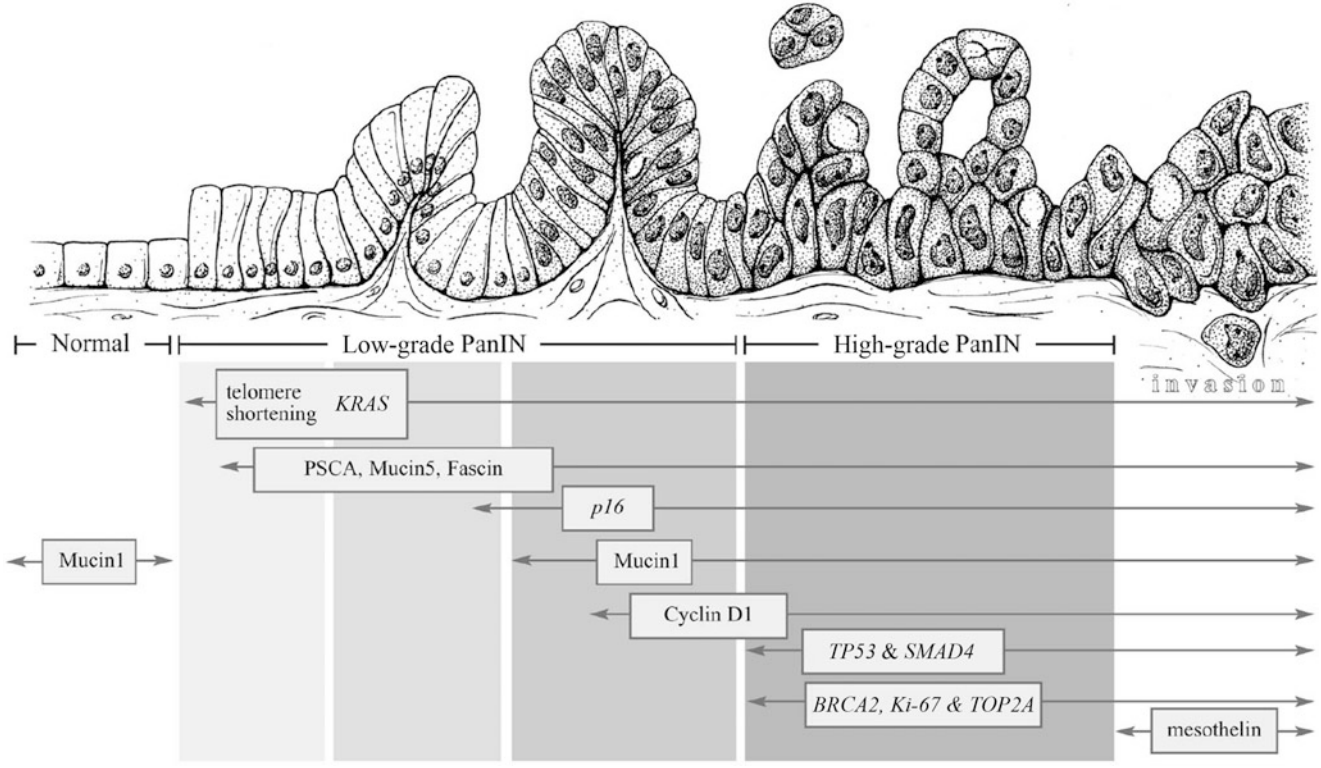

Fig. 2 Schematic illustration of some of the key genetic alterations observed during the multistep progression from the normal duct to PanIN and eventually PDAC in the form of a "PanIN-gram." The alterations shown are not comprehensive and are discussed in detail within the text (Reproduced from Maitra et al. [9] with permission)

Tumor Suppressor Gene Mutations in PanIN Lesions

Three tumor suppressor genes frequently inactivated in PanIN lesions, mirroring their common loss of function in PDAC, are *CDKN2A*, *TP53*, and *SMAD4/DPC4*. The *CDKN2A* gene on chromosome 9p21 encodes for the cell-cycle checkpoint protein p16, which binds to the cyclin-dependent kinases CDK4 and CDK6, and thereby inhibiting cyclin D1-binding and causing cell-cycle arrest in G1-S [19]. The *CDKN2A* gene is inactivated in virtually all PDACs: in approximately 40% of cases, this is due to homozygous deletion; another 40% carry intragenic mutations and show loss of the second allele; and 15% demonstrate epigenetic inactivation [20, 21]. Loss of p16 expression, which can be exploited as surrogate marker of the *CDKN2A* gene status, correlates with PanIN progression and is observed in 30–55% of low-grade PanINs and 71% of high-grade PanINs [22]. Interestingly, the frequencies of *CDKN2A* inactivation appear to be lower in PanIN lesions associated with chronic pancreatitis [23]. In a subset of cases, homozygous deletions of *CDKN2A* at 9p21 can also include homozygous deletion of the *methylthioadenosine phosphorylase* (*MTAP*) gene, whose product is required for the salvage pathway of purine synthesis. Codeletion of *MTAP* and *CDKN2A* is observed in approximately one-third of PDACs, and 10% of high-grade PanINs [24, 25].

The tumor suppressor gene *TP53* on the short arm of chromosome 17 encodes the protein p53, which plays a key role in mediating several important physiological functions, including regulation of the G1/S cell-cycle checkpoint, maintenance of G2/M arrest, and induction of apoptosis. Therefore, the inactivation of p53 in the majority of PDACs affects two major mechanisms controlling cell number: cell proliferation and apoptosis. Moreover, p53 abrogation contributes to genomic instability observed in PDACs [26]. Loss of *TP53* function is observed in 50–75% of PDAC and almost exclusively through intragenic mutations and loss of the second allele [27]. Nuclear accumulation of p53 using immunohistochemistry largely correlates with the mutational status of *TP53* and can therefore be used as a surrogate marker of *TP53* mutations in PanIN lesions. Immunohistochemistry reveals intranuclear p53 accumulation mostly in high-grade PanINs, and, thus, suggesting that *TP53* mutations constitute rather late events in the multistep pancreatic cancer progression cascade [9].

SMAD4 on chromosome 18q is inactivated in approximately 55% of PDACs by homozygous deletion in 30% of cases, or through intragenic mutation and loss of the second allele in another 25% [28]. *SMAD4* encodes the protein Smad4, which is involved in transforming growth factor (TGF)-beta signaling. The activation of the TGF-beta signaling pathway leads to binding of Smad4 to a phosphorylated Smad2/3 protein complex and its translocation to the nucleus, where it binds to specific promoter regions and induces expression of respective target genes [29]. Therefore, the loss of Smad4 function interferes with the intracellular signaling cascade downstream of TGF-beta and leads to reduced growth inhibition through loss of proapoptotic stimuli and inappropriate G1/S transition [30]. A potential alternative mechanism was recently unmasked in an elegant study showing that selective loss of Smad4-dependent signaling in T-cells leads to development of

epithelial cancers of the gastrointestinal tract in mice, while no tumor development was observed in mice with epithelial-specific deletion of *SMAD4*. These observations suggest that in addition to the abovementioned cell functions, Smad4 might also be crucially involved in interactions between cancer cells and the microenvironment and/or modulation of immune surveillance [31]. As described above for p53, immunohistochemical labeling for Smad4 can be used as a surrogate marker of the *SMAD4* mutational status [32]. Loss of Smad4 nuclear expression is observed in about one-third of high-grade PanINs, while it is preserved in normal ducts and low-grade PanINs [9, 33]. Therefore, *SMAD4* mutations, like mutations of *TP53*, represent a relatively late genetic event in the progression model for pancreatic cancer.

Caretaker Gene Mutations in PanIN Lesions

Caretaker genes comprise a third class of cancer-related genes, which are not directly involved in controlling cell growth or apoptosis, but rather help to maintain DNA integrity, e.g., by means of mismatch repair, nucleotide-excision repair, and base-excision repair [34]. By repairing subtle changes in the genomic DNA sequence that occurs due to polymerase errors or as a result of exposure to mutagens, as well as gross chromosomal aberrations, caretaker genes prevent accumulation of mutations within a cell that might provide a selective advantage leading toward a malignant phenotype.

The Fanconi anemia gene family is a group of caretaker genes known to be involved in pancreatic carcinogenesis [35, 36]. The Fanconi anemia gene family is involved in homologous recombination repair in response to DNA damage, e.g., by crosslinking agents or radiation [37]. One member of this family, the breast and ovarian cancer susceptibility gene *BRCA2* on chromosome 13q, is of particular interest in the setting of familial pancreatic cancer, since germline *BRCA2* mutations are found in 5–10% of familial cases, especially in individuals of Ashkenazi Jewish heritage [38, 39]. In addition, PDACs harboring Fanconi anemia mutations are exquisitely sensitive to DNA crosslinking agents, presenting an avenue for synthetic lethal therapy [36]. In patients with germline *BRCA2* mutations, loss of the second allele is observed in high-grade PanINs, suggesting that akin to p53 and Smad4, inactivation of BRCA2 function also constitutes a late genetic event [40].

Genomic Instability and Telomere Length Alterations in PanIN Lesions

Telomeres consist of hexameric TTAGGG repeats at the ends of chromosomal DNA strands, which confer chromosomal stability during cell division by preventing the ends from becoming sticky. Telomere attrition is among the earliest and most common alterations observed in PanIN lesions. Interestingly, significant telomere shortening is observed in over 90% of low-grade PanINs [41]. It has been speculated

that telomeres conduct a similar function to those of caretaker genes in pancreatic carcinogenesis, such that telomere dysfunction facilitates progressive accumulation of additional chromosomal abnormalities that culminates in the development of PDAC.

Reflecting their inherent genomic instability, structural and numerical chromosomal aberrations can be found in almost all cases of PDAC and often involve loss of significant proportions or the entirety of chromosomal arms. Chromosomal regions frequently involved in loss of one allele (designated loss of heterozygosity [LOH]) in PanINs include 1q, 6q, 7p, 9p, 10q, 14, 16q, 17p, and 18q [42]. Of note, the frequency of LOH observed at a given locus commonly increases from low- to high-grade PanINs. It has been proposed that LOH might in many cases be the first event in the "two-hit" cascade leading to inactivation of tumor suppressor genes. This concept is in line with the hypothesis of genomic instability beginning early in the PanIN progression model.

Epigenetic Alterations in PanIN Lesions

The most common form of epigenetic alterations found in PDAC, and also in PanIN lesions, consists of methylation of CpG islands within promoter regions, leading to transcriptional silencing of the regulated gene. Over recent years, epigenetic gene silencing – in addition to genetic alterations such as deletions and intragenic mutations – has increasingly been recognized as one of the most ubiquitous mechanisms exploited by cancer cells to alter their inherent transcriptomic programs in favor of more rapid cell growth, invasiveness, and resistance to apoptosis [43].

Current evidence supports the notion that aberrant DNA methylation occurs early during the progression of pancreatic cancer. Using a gene candidate approach, Rosty et al. demonstrated that PanIN lesions in patients with chronic pancreatitis show the loss of p16 expression, suggesting that this alteration may contribute to the predisposition of patients with chronic pancreatitis to develop PDAC [23]. In a large-scale methylation analysis with subsequent validation via methylation-specific PCR, Sato et al. analyzed DNA samples from 65 PanINs for methylation status of eight genes (*ST14, CDH3, CLDN5, LHX1, NPTX2, SARP2, SPARC*, and *Reprimo*) that were identified previously through a microarray approach as aberrantly hypermethylated in PDAC [44]. Among PanINs examined in this study, methylation of any of these eight genes was identified in 68% of cases with methylation prevalence increasing from low-grade to high-grade PanIN for *SARP2*, *Reprimo* and *LHX1*. Peng et al. had examined promoter methylation patterns of 12 cancer-related genes (*p14, p15, p16, p73, APC, hMLH1, MGMT, BRCA1, GSTP1, TIMP-3, CDH1*, and *DAPK-1*) in 40 microdissected PanIN lesions and 147 discrete areas sampled from PDACs [45]. The frequency of at least one methylated gene locus increased significantly from normal ductal epithelium lacking signs of inflammation to PanINs, and from PanINs to PDAC, respectively, further underscoring that epigenetic progression is also a feature of the traditional "PanIN-gram" model. Determination of aberrantly methylated gene promoters in pancreatic juice samples

has emerged as a potential diagnostic tool for PDAC and its precursor lesions, with a suggestion that it might be more specific than detection of mutated or differentially expressed genes [46]. In particular, certain promoter sequences like that of the *TSLC1* gene are methylated only in higher-grade PanIN lesions, and therefore, might identify those lesions that pose a greater relative risk of progression to invasive adenocarcinoma.

Transcriptomic Abnormalities in PanIN Lesions

With the advent and increasingly widespread deployment of global gene expression profiling techniques, including RNA sequencing, serial analysis of gene expression (SAGE), and various forms of oligonucleotide and cDNA/miRNA microarrays, there has been a dramatic increase in our knowledge of differential gene expression patterns in PDAC [47–51]. A few compelling examples of differentially expressed genes with translational potential will be discussed here. Although initially discovered in the context of invasive cancer, the differential expression of these genes has since been validated in varying grades of PanIN lesions as well.

Prostate stem cell antigen (PSCA) is overexpressed in 30–40% of low-grade IPMNs and 60% of high-grade IPMNs, in line with PSCA upregulation being an early event in the PanIN progression model [9]. Of note, the recent pilot studies showed that PSCA overexpression might be a suitable target for the development of novel diagnostic tools for PDAC [52]. Another example is mesothelin, a membrane-bound GPI-anchored protein known to play a role in cell adhesion. Unlike PSCA, mesothelin expression was detected only in 11% of PanIN lesions, but close to 100% of PDACs, suggesting that mesothelin overexpression is a late event [9, 53]. Recent studies have examined mesothelin as an antigen for cancer cell-specific drug delivery and for cancer immunotherapy [54]. A study by Sutherland et al. using oligonucleotide microarrays described the upregulation of several components of the retinoic acid signaling pathway, including RAR-alpha, HOXB6 and HOXB2 in PDAC, as compared to the normal pancreas [55]. In particular, HOXB2 expression was identified as prognostic marker in PDAC that correlated with survival, surgical resection, and tumor stage at the time of diagnosis. Nuclear immunostaining for HOXB2 was observed in 8% of normal pancreatic ducts, 14% of PanIN lesions, and 38% of PDACs. This suggests HOXB2 overexpression increases during pancreatic carcinogenesis.

Changes in microRNA (miRNA) expression are also important in the development of PDAC. miRNAs are small endogenous noncoding RNAs of 14–24 nucleotides that negatively regulate protein expression at the posttranscriptional level by inhibiting translation and/or by targeting mRNAs for degradation. Furthermore, because miRNAs are stable and detectable in human plasma, they are being investigated for their use as diagnostic serum markers. PDACs overexpress several miRNAs including miR-21, miR-34, miR-146a, miR-155, miR-196b, and miR-200a/b [56–58]. In a large comprehensive miRNA study by Yu et al., the authors identified 107 aberrantly expressed miRNAs based on the PanIN grades

and compared with normal pancreatic duct samples [59]. Further, 35 aberrantly expressed miRNAs in high-grade PanINs compared with normal pancreatic duct samples. These differentially expressed miRNAs included those that have been previously identified in PDACs as well as miRNAs not previously described as differentially expressed in these lesions (e.g., miR-125b, miR-296-5p, miR-183*, miR-603, miR-625/*, and miR-708). Interestingly, miR-196b was the most differentially expressed miRNA in high-grade PanINs.

Cell Cycle and Proliferation Abnormalities in PanIN Lesions

Much like PDAC, PanIN lesions also demonstrate aberrations in cell cycle checkpoint control and proliferation. While low-grade PanINs are minimally proliferative, this index significantly increases in high-grade PanINs, as assessed by nuclear expression of the proliferation antigen Ki-67/MIB-1. Klein et al. described mean nuclear Ki-67/MIB-1 labeling indices as 0.41% for normal ducts, 5.7% for low-grade PanIN, and 22.0% for high-grade PanIN [60]. The average labeling index for PDACs was 37.0%, reflecting the progressive increase in proliferative potential during the progression from normal ducts to PDAC [60]. Cyclin D1 is involved in regulating cell cycle progression by acting as a cofactor in phosphorylating and inactivating the retinoblastoma (Rb) protein, and its expression has been linked to poor prognosis and decreased survival in PDAC. Overexpression of cyclin D1 is observed in 14% of low-grade PanINs, 57% of high-grade PanINs, and up to 60–85% of PDACs [9]. $p21^{WAF/CIP1}$ acts as cyclin-dependent kinase inhibitor that inhibits cyclin E/CDK2 complexes and prevents phosphorylation of Rb. Overexpression of $p21^{WAF/CIP1}$ is an early event and is observed in 33% of low-grade PanINs, 80% of high-grade PanINs, and 85% of PDACs [61].

Aberrantly Activated Growth Factor Signaling Pathways in PanIN Lesions

Cyclooxygenase-2 (COX-2) is upregulated in PDAC, possibly secondary to activation of nuclear factor kappa B signaling, and is postulated to be involved in cell proliferation and tumor angiogenesis [62]. In PanINs, COX-2 is generally found to be overexpressed in high-grade PanINs as compared to low-grade PanINs and normal ducts [63]. COX-2 inhibitors have been suggested as potential chemopreventive agents against PDAC [64], but initial clinical efficacy data have been equivocal thus far. Members of the matrix metalloproteinase (MMP) family of zinc-dependent extracellular proteinases are involved in enabling cell invasion and metastasis [65]. Overexpression of MMP-7 is observed in the majority of PDACs, as well as in greater than half of low-grade PanINs [66]. Urinary plasminogen activator (uPA) converts plasminogen into plasmin, which in turn activates MMP precursors. In addition, uPA induces the upregulation of various downstream signaling molecules, including fibroblast growth factor 2 (FGF2) and angiostatin [67, 68]. In one study, uPA

immunolabeling was observed not only in the majority of PDACs but also in 19 of 27 (70%) low-grade PanINs and 12 of 27 (44%) high-grade PanINs [69].

Aberrantly Activated Embryonic Signaling Pathways in PanIN Lesions

Embryonic signaling pathways, including Hedgehog, Notch, and Wnt, which are usually inactive in differentiated tissues of the adult pancreas, have been found to be aberrantly reactivated in PDACs as well as in a variety of other epithelial human cancers [70–72]. This finding is of particular interest, since these signaling networks might contribute to maintain specific subpopulations of cancer cells with enhanced tumor-initiating properties, often referred to as "cancer stem cells." This concept has direct translational implications, since all of the three abovementioned embryonic signaling pathways represent candidate drug targets. The phenotype of the putative cancer stem cell compartment in PDAC has recently been elucidated by multiple groups. For example, Simeone et al. have demonstrated that a subpopulation of CD44+/CD24+/ESA+ cells, which represent less than 1% of cancer cells within a "bulk" isolate, harbor more than 100-fold increased tumorigenic potential in immunodeficient mice, as compared to nontumorigenic cells. Of note, in this population they also observed a ~10-fold overexpression of the Hedgehog ligand sonic hedgehog (Shh) as compared to bulk tumor tissues [73]. Similarly, Feldmann et al. found that inhibition of Hedgehog signaling by means of small molecule inhibitors diminished tumor initiation and metastasis in orthotopic xenograft models of PDAC, mirrored by significant reduction of a subpopulation of cancer cells with high aldehyde dehydrogenase (ALDH) activity in vivo and in vitro [74]. The concept that Hedgehog signaling is involved in maintaining a "cancer stem cell niche" would imply, that Hedgehog pathway reactivation occurs very early during the carcinogenic cascade, and indeed overexpression of Shh has been observed by immunohistochemistry in low-grade PanINs, but not in normal pancreatic ductal epithelium [75]. Further evidence came from another study by Leach et al. demonstrating that low-grade PanINs express a cluster of "foregut-specific" markers, including pepsinogen C, MUC6, KLF4, GATA6, Sox-2, Forkhead-6, and TFF1, which is very similar to differential gene expression patterns observed in immortalized pancreatic ductal epithelial cells upon transfection with the Hedgehog transcription factor Gli1 [76].

Analogous to the aberrant expression of Hedgehog pathway components, murine and human PanINs and PDACs also express multiple Notch components [72]. As observed for Hedgehog signaling, Notch pathway activation during pancreatic carcinogenesis is most likely to be due to endogenous ligand overexpression, rather than mutational events. For example, the activating Notch ligand, Jagged-1, is overexpressed in low-grade PanINs [76]. The activation of Wnt signaling in cancer tissues usually occurs due to intragenic mutations, i.e., either activating *CTNNB1/beta-catenin* mutations or loss-of-function mutations within the *APC* gene, resulting in nuclear translocation of beta-catenin and subsequent transcription

of Wnt target genes [77]. In PDAC, however, canonical pathway activation is more often ligand-dependent, than through mutational events [78]. Immunohistochemical detection of nuclear beta-catenin can be used as a surrogate marker of Wnt pathway activation. Al-Aynati et al. reported nuclear overexpression of beta-catenin in a small proportion of high-grade PanINs [79], but observations regarding PDACs have been conflicting [71].

Genetically Engineered Mouse Models and Murine PanINs (mPanINs)

A remarkable advance achieved in the last decade for pancreatic cancer research has been the development of genetically engineered mouse models, which resemble cognate properties of the human disease, such as a multistep progression involving noninvasive precursor lesions culminating in lethal disseminated malignancy [13, 14, 26]. In order to distinguish precursor lesions in mice from those arising in human pancreata, the former have been designated as murine PanIN (mPanIN) [80]. Interestingly, mPanIN lesions observed in these models also harbor many of the molecular alterations found in humans, including activation of the Notch and Hedgehog signaling pathways [13, 81]. These mouse models represent a unique platform for discovery of early pancreatic neoplasia-associated biomarkers in serum, as recently demonstrated by Hanash and colleagues [82]. In this study, the investigators identified a large panel of abnormally expressed protein the sera of mice from both early and late stage disease. Of note, when five of these proteins were examined in human sera obtained from PDAC patients, they were able to predict the diagnosis of malignancy as much as 7–13 months prior to onset of clinical symptoms, underscoring the commonalities between mouse and human disease models. Genetically engineered mouse models of mPanINs and PDAC have also begun to be utilized as in vivo platforms for assessment of novel chemoprevention and treatment modalities. For example, it has been demonstrated that the COX-2 inhibitor nimesulide can downregulate mPanIN formation in genetically predisposed mice [83], an expected finding, given that mPanINs (as well as their human counterparts) overexpress COX-2 [13].

Therapeutic Implications of Isolated PanIN Lesions

Currently, the detection of PanIN lesions is hampered by the lack of sensitive noninvasive diagnostic tools. Due to their microscopic size, PanIN lesions are usually not diagnosed by standard clinical imaging techniques. Recent data from the Johns Hopkins Hospital suggest that a combinatorial approach of collecting secretin-stimulated pancreatic juice, endoscopic ultrasound (EUS), and computer tomography might enable the detection of morphological and genetic changes associated with PanIN lesions in the adjacent pancreatic parenchyma [84]. In particular, Brune et al. showed that PanINs can be associated with a lobulocentric form of atrophy in the adjacent parenchyma, and a diffuse distribution of this atrophy

observed in patients with multifocal PanIN lesions confers a diagnostic pattern on EUS [85]. Even if further improvements in imaging techniques and other diagnostic tools will provide the means to reliably and noninvasively screen for the presence of PanIN lesions, the therapeutic implications that of such findings are largely unknown. While the pathophysiological concept of a multistep progression of PanINs culminating in PDAC has become acceptable, the appropriate clinical management of noninvasively diagnosed PanIN lesions in an individual patient still needs to be defined. In an effort to estimate the approximate probability of a single PanIN to progress to cancer, Terhune et al. applied a mathematical model, assuming that PanIN lesions can be found in 37.5% of cases in a normal population with an average of five foci per affected pancreas, and that 0.8% of pancreata develop PDAC [86]. The authors argued that based on these assumptions only about 1% on PanIN lesions progress to PDAC. These considerations underscore the caution mandated in drawing therapeutic conclusions based on the identification of PanIN lesions alone, in the absence of a discernible malignancy.

Interestingly, collection of secretin-stimulated pancreatic juice has emerged as a promising adjunct to the evaluation of precursor neoplasms. Digital next-generation sequencing ("digital NGS") to detect low-abundance mutations in secretin-stimulated pancreatic juice samples collected from the duodenum in subjects with a family history of PDAC has identified low abundance of *KRAS* mutations that are thought to arise from small PanIN lesions [87]. However, further studies are needed to assess whether the genetic alterations associated with high-grade PanINs can be reliably detected by digital NGS with a high sensitivity and high specificity.

Intraductal Papillary Pucinous Neoplasms (IPMN)

Clinical Features of IPMNs

Intraductal papillary mucinous neoplasms (IPMNs) are mucin-producing epithelial neoplasms that arise from the main pancreatic duct (main duct IPMN), branches (side branch IPMN), or both (mixed main and branch duct IPMN). A population-based study estimated the age and sex-adjusted cumulative incidence of an IPMN to be 2.04 per 100,000 individuals per year [88]. In comparison, the incidence of PDAC is 0.8 per 100,000 individuals per year. These neoplasms occur more frequently in men than women with a mean age at presentation of approximately 65 years. The majority of IPMNs are identified incidentally on abdominal computed tomography (CT) or magnetic resonance imaging (MRI), but a subset can be associated with epigastric/abdominal pain, pancreatitis, weight loss, and jaundice [89, 90]. IPMNs are usually greater than 1 cm in size, commonly arise in the head of the pancreas and can be multifocal. Similar to PanINs, the neoplastic cells may show varying degrees of dysplasia that can progress from low-grade dysplasia to high-grade dysplasia and PDAC [91]. In addition, the risk of high-grade dysplasia and PDAC is higher in patients with main duct IPMN and mixed main and branch duct IPMN than branch duct IPMN (60% vs. 25%, respectively) [89, 92]. Although the

rate of progression to advanced neoplasia in an IPMN has yet to be defined, patients with a PDAC arising in an IPMN are generally 3–5 years older than those with a non-invasive IPMN [93]. Thus, it is hypothesized that there is a substantial window of opportunity to detect and treat noninvasive IPMNs before they progress to PDAC.

Histopathological Features of IPMNs

As mentioned previously, IPMNs can be subdivided into three groups based on their location with respect to the pancreatic ductal system: main duct, branch duct, and mixed main and branch duct. Interestingly, main and branch duct IPMNs differ in their clinicopathologic features (Table 2). Based on the degree of architectural and cytologic atypia, IPMNs are graded as either low-grade or high-grade. Representative histologic images of these lesions are shown in Fig. 1c, d. Main duct IPMNs have an increased frequency of harboring high-grade dysplasia and more often associated with a PDAC than branch duct IPMNs [96, 97]. The neoplastic epithelium lining the papillae can demonstrate a variety of directions of differentiation, but the biologic and clinical significance of patterns of differentiation remain controversial. Most IPMNs adopt an intestinal differentiation and resemble intestinal adenomas with well-formed, long villous projections, lined by columnar mucinous epithelium with cigar-shaped nuclei. Most of the neoplastic cells contain abundant apical mucin and, in some cases, have scattered goblet cells. Gastric foveolar differentiation is characterized by eosinophilic cytoplasm, basally oriented nuclei, and abundant apical cytoplasm mucin. Gastric foveolar type IPMNs can be papillary or flat in appearance. The pancreatobiliary type IPMN is less common and the neoplastic cells form more complex papillae with bridging and cribriform structures. The nuclei are rounder than the intestinal type and the chromatin pattern is open, often with prominent nucleoli. This type contains less apical mucin and tends to harbor at least high-grade dysplasia. The intestinal and pancreatobiliary types of IPMN more commonly arise in the main duct, while the gastric type of IPMN is usually a branch duct lesion. The histological subtypes also demonstrate different patterns of apomucin labeling, with the intestinal-type IPMNs expressing MUC2, the pancreato-biliary type expressing MUC1, and the gastric type IPMN expressing MUC5AC, but usually lacking MUC1 and MUC2. In addition to intestinal, gastric

Table 2 Clinical and pathologic features associated with main duct and branch duct IPMNs [94, 95]

	Main duct IPMN	Branch duct IPMN
Age peak	55 years	65 years
Location in pancreas	57% in head	93% in head
Dysplasia/malignancy		
Low-grade dysplasia	43%	85%
High-grade dysplasia	20%	15%
Invasive adenocarcinoma	37%	0%

and pancreatobiliary type IPMNs, there is another histologic variant that is referred to as intraductal oncocytic papillary neoplasm (IOPN). The neoplastic cells found within IOPNs show abundant eosinophilic cytoplasm, due to the high number of mitochondria in these cells. However, whether IOPNs should be classified as a subtype of IPMNs or a distinct entity remains controversial.

IPMNs can be associated with two predominant subtypes of PDAC that include colloid (mucinous noncystic) carcinoma and conventional ductal adenocarcinoma [98]. Distinguishing the subtypes of PDAC is clinically important, since colloid carcinomas carry a significantly better prognosis [99]. Great care should be taken not to overlook an associated focal carcinoma, particularly because the neoplastic epithelium in an IPMN can extend intraductally for several centimeters beyond the grossly dilated duct. Of note, patients with IPMNs show an increased risk for extrapancreatic malignancies. In particular, higher rates of colorectal, gastric, esophageal, and lung malignancies have been reported [100].

Molecular Features of IPMNs

Studies have identified a variety of genetic alterations in IPMNs. The most frequent genetic alteration is an oncogenic *KRAS* mutation, which has a prevalence of >80%. *KRAS* encodes for a G-protein, or a guanosine-nucleotide-binding protein, that functions as a small GTPase and mediates downstream MAPK/ERK signaling from growth factor receptors [101, 102]. Missense mutations result in constitutive activation of *KRAS* and occur primarily in codon 12 and, to a lesser extent, codons 13 and 61 [101]. *KRAS* mutations are detected in all histologic subtypes of IPMNs, but are more likely present in the gastric and pancreatobiliary types. Further, Nikiforova et al. found *KRAS* mutations in IPMNs are associated with a branch duct location [101]. In addition to *KRAS*, 65% of IPMNs harbor somatic mutations in the oncogene *GNAS*, which encodes for the G-protein stimulating α subunit (Gsα) [102]. Mutations in *GNAS* at either codon 201 or 227 result in constitutive activation of Gsα and its effector adenylate cyclase, leading to autonomous synthesis of cAMP and uncontrolled growth signaling [102, 103]. *GNAS* mutations are more often present in IPMNs involving the main pancreatic duct than branch duct, and of an intestinal histologic subtype. Collectively, activating mutations in *KRAS* and/or *GNAS* are present in >96% IPMNs and considered early genetic events in the progression to PDAC.

In addition to *KRAS* and *GNAS*, inactivating mutations in the tumor suppressor gene *RNF43* occur in 14–38% of IPMNs with frequent loss of heterozygosity [104, 105]. *RNF43* encodes for an E3 ubiquitin ligase that regulates the Wnt signaling pathway. Similarly, activating mutations in *CTNNB1* also occur in small subset of IPMNs [106]. Other potential genes mutated in IPMNs include *TP53*, *PIK3CA*, *PTEN*, *CDKN2A*, and *SMAD4*. *TP53* mutations occur late in the neoplastic progression of IPMNs and are frequently seen in advanced neoplasia [107]. Similarly, Garcia-Carracedo et al. found *PIK3CA* mutations and deletions in *PTEN* are strongly associated with high-grade IPMNs and PDAC [108, 109]. Losses in *CDKN2A* are an

uncommon finding, but more prevalent in IPMNs with high-grade dysplasia than low-grade dysplasia [110, 111]. *SMAD4* is also rarely inactivated in low-grade IPMNs, but mutations with corresponding loss of heterozygosity are typically seen in the setting of advanced neoplasia. More recently, Hata et al. demonstrated an elevated telomerase activity, presumably due to *TERT* promoter mutations, in IPMNs is more often seen in IPMNs with high-grade dysplasia and/or invasive adenocarcinoma [112].

Epigenetic silencing by aberrant promoter methylation has been described for a number of candidate tumor suppressor genes in IPMNs, including *SOCS1, ppENK, CDKN1C*, and *CDKN2A* [113, 114]. In recent years, several studies have uncovered a plethora of differentially expressed genes in IPMNs. Transcripts found to be overexpressed in IPMNs that represent candidate biomarkers, and which might also potentially be involved in IPMN progression, include *lipocalin-2, galactin-3, cathepsin-E, claudin-4, TFF-1, TFF-2, TFF-3, CXCR-4, S100A4, matrix metalloproteinase 7 (MMP-7),* and *sonic hedgehog (SHH)* [115–117]. The recent availability of technologies that can enable mass spectrometric based approaches on microdissected tissues has enabled one of the first global proteomic analysis of a noninvasive IPMN [118]. This study, using microdissected material from an archival IPMN, identified tissue transgluaminase-2 (TTG-2) and deleted in malignant brain tumor 1 (DMBT1) as candidate biomarkers in these precursor lesions.

Genetically Engineered Mouse Model of IPMNs

In an elegant study, Schmidt and coworkers described that concomitant pancreas-specific expression of an oncogenic *Kras* allele and transforming growth factor-alpha (TGF-alpha) led to formation of acinar-ductal metaplasia, accelerated progression of *Kras*-induced mPanINs, as compared to *Kras* expression alone, and to the development of cystic lesions resembling key features observed in human IPMNs starting at 2–3 months after birth [119]. Histologically, these cystic lesions were characterized by papillary proliferations which had formed in branches of the main pancreatic duct. In line with findings in humans, the observed murine IPMNs were shown to express CK19, MUC1 and MUC5AC.

Studying the potential role of *GNAS* in pancreatic carcinogenesis, Taki et al. generated transgenic mice that included activated *GNAS* [120]. These mice showed elevated cAMP levels, small dilated tubular complex formation, loss of acinar cells, and fibrosis in the pancreas; but, no macroscopic tumorigenesis was apparent by 2 months of age. However, the combination of *KRAS* and *GNAS* resulted in mice developing cystic tumors consisting of markedly dilated ducts lined by papillary dysplasia epithelium in the pancreas that closely mimicked human IPMNs.

Interestingly, mutations in *Brg1* and other members of the SWI/SNF complex have been observed in over 30% of PDACs, and decreased Brg1 protein expression has been identified in a subset of IPMNs [121]. Inactivation of *Brg1* in combination with mutant *Kras* in mice promoted the development of cystic neoplastic lesions that resemble

IPMNs and over time progress to PDAC [122]. These findings suggest that chromatin remodeling may underlie the development of IPMNs and the formation of PDAC.

Therapeutic Considerations Regarding IPMNs

The major clinical challenge with IPMNs is differentiating IPMNs with high-grade dysplasia and PDAC from IPMNs with low-grade dysplasia. Moreover, another clinical conundrum is predicting whether an IPMN will follow an indolent or malignant disease course. As a consequence, a number of consensus- and evidence-based management and treatment guidelines have been developed for IPMNs and heavily rely on cross-sectional abdominal imaging, endoscopic ultrasound, and pancreatic cyst fluid ancillary studies, such as carcinoembryonic antigen (CEA) and cytopathology [89, 123, 124]. However, these diagnostic modalities have clear limitations in predicting malignancy with a high sensitivity and high specificity. Thus, there has been a growing interest in identifying molecular markers to guide management for IPMNs.

In a pilot study, Khalid et al. prospectively evaluated the presence of mutations in *KRAS* and allelic imbalance in seven tumor suppressor genes by Sanger sequencing in preoperative pancreatic cyst fluid [125]. The authors found the combination of *KRAS* mutations and allelic loss to be predictive of advanced neoplasia within an IPMN with a sensitivity and specificity of 91% and 93%, respectively. These results were later expanded into a multicenter prospective study (Pancreatic Cyst DNA Analysis Study or PANDA study) of 113 patients [126]. Pancreatic cyst fluid was collected preoperatively by EUS-fine needle aspiration (FNA) and assessed for *KRAS* mutations and the overall fraction of alleles lost compared to germline DNA (mean allelic loss amplitude or MALA).

In the PANDA study, the presence of mutant *KRAS* alone had a sensitivity and specificity of 45% and 96%, respectively, for a mucinous cyst, but was not predictive of advanced neoplasia. In contrast, a high MALA (>82%) had 90% sensitivity and 67% specificity for advanced neoplasia. But there were a number of weaknesses in the study design that diminished the overall significance of these results. Notably, it was unclear if DNA analysis would add value to established pancreatic cyst management guidelines. Furthermore, there was concern that MALA may be confounded by DNA degradation, gastrointestinal contamination during EUS-FNA, and other variables. Indeed, follow-up studies demonstrated broad variability in agreement between molecular and clinical diagnoses. Shen et al. reported an 89% concordance between molecular and clinical consensus diagnoses, while Panarelli et al. and Toll et al. reported a concordance rate of 39% and 56%, respectively [127–129].

Regardless of the issues with MALA, *KRAS* testing proved to be a cost-effective strategy to identify patients with IPMNs and MCNs. In a cohort of 618 patients, Nikiforova et al. found mutant *KRAS* had 54% sensitivity and 100% specificity for a mucinous cyst [101]. This assay was superior to CEA testing and utilized significantly less pancreatic cyst fluid for analysis. Moreover, the combination of *KRAS*

point mutations and elevated CEA improved the sensitivity to 83% and maintained a high specificity of 85%. The sensitivity of molecular analysis for mucinous cysts was further increased by the addition of *GNAS* testing. Singhi et al. showed the detection of mutant *KRAS* and/or *GNAS* had a sensitivity and specificity of 65% and 100%, respectively [103]. However, there was significant discordance in the rates of detection of *KRAS* and *GNAS* mutations between preoperative EUS-FNA and studies using postoperative pancreatic cyst fluid. The authors underscored the limitations of their assay may be due to the inherent sensitivity and specimen requirements of conventional Sanger sequencing.

The limit of detection of Sanger sequencing is approximately 15–20% of mutant alleles. In comparison, next-generation sequencing (NGS) has a limit of detection of approximately 3–5% of mutant alleles. Recent studies have shown the application of NGS to pancreatic cyst fluid ranges from 86% to 90% in sensitivity and 75% to 100% in specificity for mucinous differentiation [130, 131]. Other advantages of NGS are the small amounts of DNA required for analysis and the ability to assay multiple genes simultaneously. Using a broad panel of genes to include *KRAS*, *GNAS*, *VHL*, *TP53*, *CDKN2A*, and *SMAD4*, among others, Jones et al. identified a high concordance rate between molecular and clinical diagnoses [131]. Similarly, Singhi et al. found mutations in *TP53*, *PIK3CA*, and/or *PTEN* to have 83% sensitivity and 97% specificity in detecting advanced neoplasia within an IPMN [130]. However, as diagnostic DNA testing of pancreatic cyst fluid continues to evolve, questions remain as to how these alterations will influence patient management.

Mucinous Cystic Neoplasms (MCN)

Clinical Features of MCNs

Mucinous cystic neoplasms (MCNs) are also mucin-producing epithelial neoplasms that arise outside of the large ducts of the pancreas. The exact incidence and prevalence of MCNs is difficult to assess, but within a large surgical series represented, MCNs comprise a quarter of all resected cystic neoplasms of the pancreas. Over 90% of MCNs are diagnosed in females, and the mean age at diagnosis is between 40 and 50 years, with a wide range described in the literature (14–95 years) [132, 133]. Not surprisingly, patients presenting with noninvasive MCNs tend to be 5–10 years younger on average as compared to those carrying MCNs with associated invasive carcinoma, in line with the concept of MCN being a precursor lesion eventually progressing to PDAC. Clinical symptoms are often unspecific and include epigastric pain, a sense of abdominal fullness and abdominal mass. Carcinoembryonic antigen 19-9 (CA19-9) blood concentrations are usually normal in noninvasive MCN patients and elevated only in cases that are associated with a PDAC [134]. Of note, MCNs, like IPMNs, can be discovered as incidental cystic lesions of the pancreas. Computed tomography typically reveals a relatively large (up to 10 cm) intrapancreatic cystic mass. Intramural nodules are more common in MCNs with an associated invasive adenocarcinoma. The cysts

themselves are usually 1–3 cm in diameter and divided by fibrous septa, cyst contents vary from mucoid to hemorrhagic fluid. MCNs do not communicate with the pancreatic duct, and this feature is often exploited to differentiate MCNs from IPMNs in the clinical setting.

Histopathology of MCNs

The cysts of MCNs are lined by a columnar mucin-producing epithelium, associated with a spectrum of architectural and cytologic atypia, akin to what is observed in IPMNs. MCNs with low-grade dysplasia consist of uniform columnar cells with abundant supranuclear mucin. The nuclei are typically uniform, small, and basally located basally (Fig. 1e). In contrast, MCNs with high-grade dysplasia demonstrate significant degree of architectural and cytologic atypia, similar to what is seen in high-grade PanINs and high-grade IPMNs [4, 132] (Fig. 1f). In addition to neoplastic epithelium, MCNs comprise a distinct "ovarian-type" stroma [133, 135]. This ovarian-type stroma consists of densely packed spindle-shaped cells, which can in some cases even show luteinization, and that form a band directly underneath the neoplastic epithelium. Per the current consensus definition, the ovarian-type stroma is an essential prerequisite for the diagnosis of an MCN. Therefore, a proportion of lesions previously referred to as MCNs are now categorized as IPMNs, and the ratio of MCNs relative to IPMNs tends to decrease in newer reports. Diagnostically, ovarian-type stroma can be particularly useful for MCN samples where the neoplastic epithelium is focally denuded. Around one-third of resected MCNs are found to be associated with PDAC [132]. These carcinomas may arise focally in an MCN, and the extent of invasion has been shown to be one of the most important prognostic factors [133].

Molecular Genetics of MCNs

The genetic alterations found in MCNs are similar to those in IPMNs. Analogous to IPMNs, activating *KRAS* mutations are the most common finding, but their prevalence increases with the degree of dysplasia. Jimenez et al. detected KRAS mutations in 26% of low-grade MCNs, while in 89% of MCNs with advanced neoplasia [136]. *RNF43* alterations are also present in MCNs and range from 8% to 35% [104, 105]. In addition, mutations and/or deletions in *TP53*, *PIK3CA*, *PTEN*, *CDKN2A*, and *SMAD4* are detected in MCNs with advanced neoplasia. However, in contrast to IPMNs, *GNAS* mutations are distinctly absent in MCNs [102, 104]. Moreover, recent studies on global expression profiling of MCNs have uncovered tissue specific overexpression of a variety of proteins. Among others, c-met, S100P, prostate stem cell antigen (PSCA), jagged-1, c-myc, cathepsin E, and pepsinogen C were found to be overexpressed by neoplastic epithelial cells, and steroidogenic acute regulatory protein (STAR) and estrogen receptor-1 (ESR-1) by ovarian-type stroma cells, respectively [137, 138].

Genetically Engineered Mouse Models of MCN

Within the past decade, at least two genetically engineered mouse models have been described, closely resembling key features of human MCNs. Mao et al. reported that the activation of the Hedgehog signaling pathway through overexpression of a mutationally activated smoothened allele (R26-Smo-M2) in mice led to the rapid development of rhabdomyosarcomas, basal cell carcinomas, and medulloblastomas [139]. Of interest, they also observed the development of a novel form of pancreatic lesions resembling low-grade MCNs in approximately half of tamoxifen-induced mice. These lesions were characterized by cyst formation of varying size, lined by cuboidal epithelium with foci of columnar metaplasia and by a supporting proliferative ovarian-like stroma. Moreover, PAS and Alcian blue stains indicated mucin expression by the epithelial cells within these lesions. Izeradjene et al. described that pancreas-specific expression of oncogenic *Kras* in combination with *Smad4* haploinsufficiency led to the formation of macroscopically visible cystic lesions in the body and tail of murine pancreata [140]. Histopathological examination revealed formation of low-grade mPanINs as well as cystic lesions resembling histological features of human MCNs, including lining by a neoplastic epithelium consisting of columnar, mucin-filled, CK19 positive epithelial cells displaying focal areas of low to high-grade dysplasia, as well as a surrounding stroma that was frequently very cellular and contained spindle-shaped cells with distinctive "wavy" nuclei. Interestingly, the cysts did not seem to communicate with the duct system.

Therapeutic Implications of MCNs

The prognosis of MCNs depends largely on whether or not there is an associated adenocarcinoma and the extent of adenocarcinoma invasion. If a PDAC is not diagnosed after thorough histopathological evaluation of a surgically completely resected MCN, the patient has an excellent prognosis and can be considered as cured [133]. If, on the other hand, a surgically resected MCN is found to be associated with an invasive carcinoma, patients show a worse 5-year overall survival of only about 60%, which is, nevertheless, still considerably better than survival rates observed for PDAC that are not associated with an MCN [133]. However, the extent of invasion of the adenocarcinoma largely dictates prognosis. Both Crippa et al. and Lewis et al. found intracapsular PDAC as defined by invasion that did not go beyond the wall of the MCN to be associated with an excellent prognosis [141, 142]. Between both studies, only 4 of 30 (13%) adenocarcinomas with intracapsular invasion recurred. Similarly, Zamboni et al. reported three patients with intracapsular invasion and five cases with extracapsular invasion. All three patients with intracapsular invasion were alive and well after a mean follow-up of 22 months [133]. In contrast, two of three patients with extracapsular invasion died of disease.

At least two clinically relevant conclusions can be drawn from these observations: First, the striking difference in prognosis between MCN with and without accompanying PDAC underscores the importance and potential of early detection

and resection of these precursor lesions. Unlike noninvasive IPMNs, MCNs are typically unifocal and represent surgically curable lesions even if they are associated with high-grade dysplasia and minimally invasive adenocarcinoma at the time of diagnosis. The observed age difference of patients with and without associated adenocarcinoma further indicates that there is probably a sufficient time window of probably several years in a given patient, before an existing MCN develops an invasive adenocarcinoma, and during which early detection and resection with curative intent are possible. Secondly, pathologists need to carefully and entirely sample MCNs for histopathologic review to adequately assess for the presence of a PDAC and document the extent of invasion.

Conclusion

In summary, PanINs, IPMNs, and MCNs represent three well-defined precursor lesions of PDAC. In the last decade, significant progress has been made in understanding their molecular genetics, development of animal models, and improvements in early detection of these lesions in asymptomatic individuals. Further advances in early detection and possibly chemopreventive clinical trials are expected to occur within the next decade and are essential in the fight against pancreatic cancer.

Box 1 Key Research Points

Three types of precursor lesions are recognized that can progress to invasive adenocarcinoma of the pancreas – pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasm (IPMN), and mucinous cystic neoplasm (MCN). Over the past decade, consensus histopathological criteria have been established that facilitate the accurate diagnosis and classification of these precursors, and permit comparable data to be generated between different institutions. The multistep progression from early to later stages of these precursor lesions is mirrored by a series of accumulating genetic alterations.

Box 2 Future Scientific Directions

While potent therapeutic options for established PDAC are lacking accounting for its overall dismal prognosis, the precursor lesions of PDAC (e.g., PanINs, IPMNs, and MCNs) represent a unique therapeutic opportunity for curative intervention. Future research should be aimed at developing diagnostic and imaging tools which allow for reliable early detection of these precursor lesions in a clinical setting. This is particularly desirable for PanINs, which are by far the most frequently observed precursor lesions and are difficult or close to impossible to detect with current clinically available imaging

(continued)

Box 2 Future Scientific Directions (continued)
techniques. Moreover, prospective studies should address individual risk estimation of diagnosed precursor lesions to enable evidence-based guidelines for the appropriate clinical management in individual cases.

Box 3 Clinical Implications
Early detection of precursor lesions of PDAC has the potential to identify high-risk patients and treat a pancreatic lesion before it progresses into a frank malignancy. The clinical implications for some precursor lesions are more obvious than others. MCNs should always be resected and thoroughly evaluated histopathologically for the presence of an associated PDAC. The same holds true for main duct type IPMNs. However, there are currently opposing opinions as to the management and treatment of branch duct type IPMNs. PanINs are a common finding in the elderly population, but to date appropriate tools to reliably diagnose isolated PanINs in a clinical setting are lacking. Recently, endoscopic ultrasound has enabled the diagnosis of multifocal PanIN lesions in patients at risk for developing PDAC (e.g., individuals with a familial pancreatic cancer). Improvements in imaging strategy and the incorporation of molecular techniques in the diagnosis and workup of precursor lesions should facilitate improved therapeutic decision making.

Cross-References

- ▶ Animal modeling of Pancreatitis-to-Cancer Progression
- ▶ Developmental Molecular Biology of the Pancreas
- ▶ Epigenetics and Its Applications to the Progression Model of Pancreatic Cancer
- ▶ Molecular Pathology of Carcinomas of the Ampullary/Periampullary Region
- ▶ Molecular Pathology of Pancreatic Endocrine Tumors

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Epigenetics and Its Applications to the Progression Model of Pancreatic Cancer

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Abstract

Defined as heritable changes in gene expression, which are not due to any alteration in the DNA sequence, epigenetic pathways have come to the forefront of research in disease, and in particular, cancer. In fact, these pathways are more prevalently altered in cancer than genetic alterations and most important, can be reversible, lending themselves as attractive therapeutic targets. This chapter will cover the basic aspects of transcriptional gene regulation, epigenetics, and chromatin dynamics and then focus on the intricacies of its application to pancreatic cancer biology and potential therapeutics. In addition, a model for better understanding pancreatic cancer is outlined to expand the highly provocative and productive "mutation centric" progression model, as defined by Hruban and colleagues, into a current model that formally includes chromatin-induced and noncoding RNA-induced epigenetic changes, as well as other alterations that result from changes in nuclear shape. This model offers a compass for further considerations aimed at illuminating the field of pancreatic cancer biology, diagnosis, therapeutics, and chemoprevention, in a similar, prolific manner as the original model.

Keywords

Epigenetics · Transcription · Chromatin dynamics · DNA methylation · Histone · Non-coding RNAs · Nucleus · Nuclear shape · Pancreatic cancer

Introduction

The phenomenon of epigenetics involves the regulation of gene expression via chromatin modifications and remodeling. Interestingly, an embryo is defined as human by the amount and sequence of DNA, which result from the fusion of the two parental gametes. However, as the embryo grows, cells will begin to differentiate from each other with this same amount and sequence of DNA. The ultimate results of the differentiation process seen in a young adult clearly show that despite all cells within the same organism carrying the same DNA sequence, a neuron, for instance, is totally different than a pancreatic acinar cell. Meditating on this phenomenon can leave one breathless. If one supposes that these two cells are independent unicellular organisms instead of both originating from a human, it would not be apparent that they have the same genome. Epigenetic mechanisms are responsible for defining cell phenotypes during the differentiation process by modulating the expression of the same genome in a different manner that is inheritable in each somatic cell division. Therefore, this chapter will (1) review the basic aspects of molecular mechanisms that are important for understanding gene regulation and epigenetics; (2) discuss the current model for better understanding pancreatic cancer,

which expands the extremely provocative and productive "mutation centric" model defined by Hruban et al. in 2000 [1] into one that formally includes chromatin-induced and noncoding RNA-induced epigenetic changes, as well as other modifications that result from alterations in nuclear shape; and (3) briefly consider drugs that may be important for the chemoprevention and/or treatment of pancreatic cancer.

Basic Concepts in Epigenetics

The study of epigenetics has been an example of how applicable the epistemological concepts behind the Thomas Kuhn's seminal work, "The Structure of Scientific Revolutions," are to this science [2]. In this work, Kuhn proposes that science moves ahead not by the incessant generation of data, but by work that changes preexistent paradigms. This is sometimes referred to as an epistemological fracture, meaning that the conceptual framework that was valid yesterday has evolved into a new theoretical framework that better explains reality. Therefore, the basis of epigenetics will be discussed through the progression of paradigms that have dominated this science at different stages of its development until today. These basic paradigms should be integrated into a picture of how chromatin and the transcriptional regulatory machinery work together in order to mediate epigenetic inheritance in somatic cells.

Evolving Paradigms in the Field of Transcription, Chromatin, and Epigenetics

The Universality of Promoters

This is the story of a remarkable journey since the work of Jacob and Monod [3] to the large amount of work that went into discovering the transcriptional mechanisms that regulate basal levels of expression before either activation or repression can occur (Basal Transcription). Prokaryote cells have only one RNA polymerase that binds to the promoter of genes and, aided by a transcription factor (factor σ), initiates the synthesis of an RNA molecule (Transcription) (reviewed in [4]). A remarkable finding is that promoters from bacteria to human contain similar sequences (e.g., TATA box). This concept has supported the prediction that the regulation of gene expression throughout evolution has been mechanistically very similar. This level of similarity was remarkable in its time, but was distant from the entire actuality. Hardcore evidence for the functional evolutionary-conservation thinkers has been further supported by the discovery that, at the atomic resolution, the tridimensional structure among RNA polymerases is strikingly high [5]. Thus, this theoretical framework paved the way for the search of eukaryote molecules that mediate transcription.

The RNA Pol II Components and the General Transcription Factors

The discovery of an RNA polymerase from eukaryotic cells highly stimulated studies aimed toward understanding transcriptional regulation [6]. However, the complexity of eukaryotes became apparent in comparison to bacteria, in particular, with the isolation of two additional RNA polymerases from higher organisms for a total of three RNA polymerase molecules, referred to as RNA polymerase I, RNA Polymerase II, and RNA polymerase III (reviewed in [7]). The intricacies of the eukaryotic system became further evident upon attempts to reconstitute transcription from isolated RNA polymerase II complexes bound to the core promoter of genes involved in basal transcription [8]. Transcription initiation at RNA polymerase II promoters in eukaryotes, which is the focus of the current chapter due to its association with protein-encoding gene expression, involves the assembly of a megadalton, multiprotein complex, comprised of the polymerase itself, as well as a variety of associated factors, known as the General Transcription Factors (GTFs). These general transcription factors function to properly position RNA pol II on the promoter DNA and to interact with transcriptional activators. The isolation and reconstitution of transcription in vitro to derive the resultant theoretical framework required several decades, until the details of the paradigm described in the following paragraph emerged.

The Step-Wise Assembly of the RNA Pol II Complex Versus the Holoenzyme Complex

To focus on the process of transcriptional initiation, it is most logical to begin with a description of RNA polymerase II complex, the transcriptional enzyme complex, responsible for making the protein-encoding RNA molecules, which includes the general transcription factors. Two paradigms exist for initiation of promoter occupancy by the RNA pol II complex: individual general transcription factors and the enzyme may be assembled in situ on the promoter in a step-wise fashion or the entire machinery and its associated factors bind the promoter collectively as the pre-assembled polymerase II holoenzyme (reviewed in [9]). Based on the step-wise assembly paradigm, the eukaryotic core promoter serves as a platform for the assembly of the transcription preinitiation complex (PIC). PIC assembly commences with TFIID binding to the TATA box, initiator, and/or downstream promoter element (DPE) present in most core promoters. The concept of the PIC was originated primarily from results of in vitro reconstitution assays, which subsequently led to the isolation of the GTFs that enter into the process of transcription in a step-wise manner to aid RNA polymerase II. These proteins include, in order of association to the promoter, TFIID, TFIIB, TFIIA, TFIIF, TFIIE, and TFIIH (reviewed in [10]). TFIID, the initial GTF to bind for PIC formation, is the only GTF with site-specific DNA binding ability and in itself a complex containing the TATA-binding protein (TBP) and numerous TBP-associated factors, termed TAF_{II}s. Subsequently, TFIIB recognizes the TFIID-promoter complex and, along with TFIIA, stabilizes the

nucleoprotein complex, which allows TFIIF to escort RNA pol II to the promoter. The interaction between TFIIB and RNA pol II is crucial for defining the proper start site of transcription [11]. Once RNA pol II is stably positioned, it is unable to initiate RNA transcription until the recruitment of two additional GTFs, TFIIE, and TFIIH. Transcriptional initiation requires two functions of the TFIIH, a helicase activity to open the double stranded DNA since the RNA polymerase will copy only a single strand of a gene, and a CDK kinase activity, which hyperphosphorylates the tail of the RNA pol II molecule to initiate transcription.

Two major discoveries have been the existence of the Mediator Complex [12], which is necessary for full function of the RNA pol II, as well as the possibility that the RNA pol II enzyme, GTFs, and Mediator could be preassembled to form the RNA polymerase II holoenzyme (enzyme with all the parts) prior to promoter recruitment. This process forms the basis of the holoenzyme paradigm [9]. The knowledge derived from both the step-wise assembly and the holoenzyme paradigm is currently operational.

The Promoter-Bashing Paradigm, Cis-Regulatory Sequences, and Sequence-Specific Transcription Factors

At the same time experiments were actively underway to understand the mechanisms regulating basal transcription, other investigators were searching for the basis of regulated transcription, namely, transcriptional activation (gene induction) and/or transcriptional repression (gene silencing). For this purpose, investigators adopted concepts and tools to dissect this process, including fusing promoter regions to reporter genes and performing deletions and site-directed mutagenesis for teasing out potential sites that could bind sequence-specific transcriptional regulators, which provided fruitful information as the promoter-bashing paradigm. In addition, promoter footprinting and Electrophoretic Mobility Shift Assays (EMSAs) were utilized to determine transcription factor binding to specific DNA sequences, called cis-regulatory sites [13]. These factors act either as monomers, such as the pancreatic tumor suppressor, and sequence-specific transcription factor, KLF11 [14], or as a complex, such as PTF1 [15], which recognizes the promoters of many acinar cell genes in a trimeric homeodomain complex including P48 and HEB. Some of this knowledge not only advanced the concept of transcription, but also generated useful tools for the Pancreatology field, since several tissue-enriched or developmental time-specific promoters (reviewed in [16]) are the key requirement for the creation of several animal models for pancreatitis and cancer.

The Coactivator-Corepressor Hypothesis

Studies designed to better decipher the way that sequence-specific transcription factors regulate gene expression led to the concept that these proteins behave as adaptors between the DNA and proteins that either induce or impede RNA pol II

transcription. This concept was based upon the recognition this type of transcription factor was modular in structure, composed of a DNA binding domain and a transcriptional regulatory domain to influence the rate of mRNA synthesis (reviewed in [17–19]). Conceptually, proteins responsible for promoting activation were called coactivators, while any corresponding repressor proteins were termed corepressors. Initially, some investigators searched for these factors among the hundreds of proteins that form the RNA Pol II holoenzyme. Indeed, interactions of transcription factors with certain members of the holoenzyme were necessary for regulated transcription. However, at the same time, a new era in studying the role of chromatin proteins was being born and starting to dominate, at the mechanistic level, the field of gene expression and apoptosis, proliferation, senescence, stem cell biology, cell migration, oncogenesis, tumor suppression, DNA replication, DNA repair, ploidy, as well as other processes integrally associated with the development and maintenance of the pancreatic cancer phenotype. For instance, it is now known that histone deacetylases (HDACs) play significant regulatory roles in gene expression during cancer [20], in particular in silencing tumor suppressor genes, and select inhibitors of these proteins are approved for clinical use in lymphoma and multiple myeloma and others are in various phases of clinical trials for the treatment of diverse malignancies [21]. HDACs are recruited into different protein corepressor complexes, which are brought to promoters via the transcriptional regulatory domain of a distinct transcription factor bound to DNA (reviewed in [22]). As a result, this transcription factor effectively deacetylates histones, which serves as a signal for gene silencing (Fig. 1). The reversal of this state is achieved through the function of histone acetylases enzymes (HATs), such as CREB binding protein (CBP)/P300 and P300/CBP-Associated Factor (PCAF) (reviewed in [23]). The deregulation of these types of enzymes leads to the aberrant activation of oncogenes (Fig. 2). Other nonhistone chromatin proteins function either as coactivators or corepressors via distinct mechanisms, as mediators of histone methylation, ubiquitination, sumoylation, and other modifications, which inform the cell toward dynamically changing gene expression patterns according to the corresponding function.

Chromatin Dynamics Forms the Basis of Epigenetics

Work on the role of histones in nuclear cell biology was very active in the 1970s with a detailed analysis of nucleosome composition and DNA packaging [24]. In terms of transcription, histones and nucleosomes were believed to be rich solely in heterochromatin, which is transcriptionally silent, and relatively poor in euchromatin, which is transcriptionally active. Unfortunately, however, how these states could be interchanged, meaning that chromatin was more dynamic than previously speculated, remained poorly understood until the 1980s and received a boost at the turn of the century (reviewed in [25]). Research on transcriptional regulation and its relevance to biological and pathobiological processes grew significantly with the discovery that indeed, chromatin is dynamic, often switching from euchromatin to heterochromatin and vice versa. Chromatin dynamics is regulated by (a) signaling

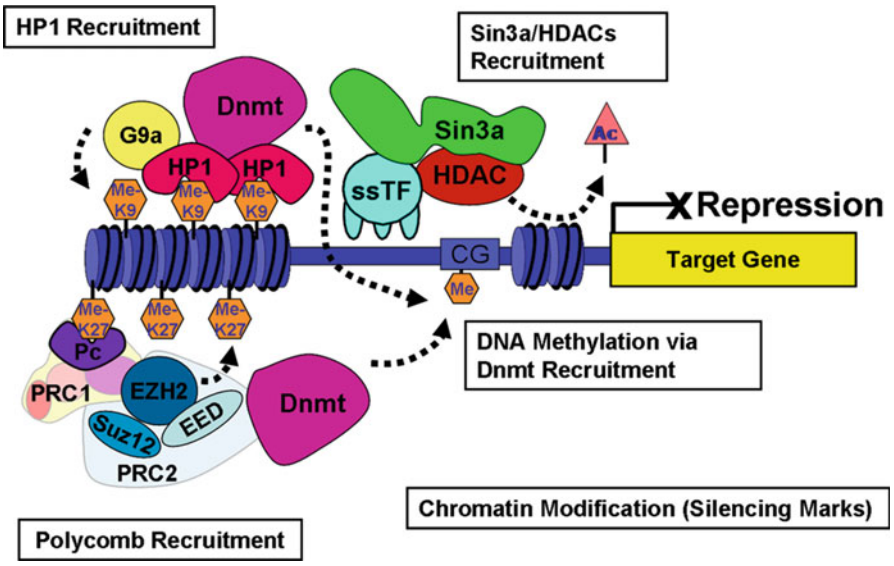

Fig. 1 Examples of Epigenetic-Mediated Tumor Suppressor Gene Silencing. This cartoon depicts a model for various roles of chromatin dynamics in tumor suppressor gene silencing, participating in the cancer phenotype. Several different mechanisms of epigenetic-mediated gene silencing can accomplish the same outcome of tumor suppressor gene silencing, including the HDAC system, polycomb proteins, and HP1 proteins. For example, a sequence-specific transcription factor (ssTF) may recruit the Sin3a-HDAC complex to a target gene promoter. The recruitment of Sin3a-HDAC to the promoter facilitates the remodeling of surrounding chromatin with silencing marks, namely the deacetylation of histones. Removal of acetylation signals short-term repression of a target gene and in addition, primes the histone for receiving additional long-term silencing marks, such as methylation of K9 or K27 on histone H3, binding marks for HP1 and polycomb, respectively. The recruitment of HP1 to a gene promoter facilitates the further recruitment of the G9a methylase, which creates more methyl-H3K9 silencing marks and thus, more HP1 binding sites. In addition, HP1 can recruit a DNA methyltransferase (Dnmt) to the promoter. In a similar manner for the polycomb group proteins, PRC1 recruitment results in the binding of the PRC2 complex, which contains the H3K27 methylase EZH2. The PRC2 complex also is capable of recruiting the DNA methyltransferases

events that form the basis of the histone code and subcodes, (b) mechanochemical enzymes that move nucleosomes from cis-regulatory sequences, an essential step in transcription, as well as (c) histone chaperones, which remove histones from nucleosomes to either activate or silence gene expression. Noteworthy, chromatin dynamics determines the epigenetic inheritance of a phenotypic trait either from the germ line (imprinting) or from one somatic cell to its daughter. DNA content is the same throughout the body, yet different types of cells with distinct characteristics and functions exist to create various organs and biological systems. Often not considered, the exact same DNA is in every cell, and thus, the distinction in the type of cell it becomes lies within epigenetics, and in particular, chromatin dynamics. Following, these three areas of chromatin dynamics are described in further detail.

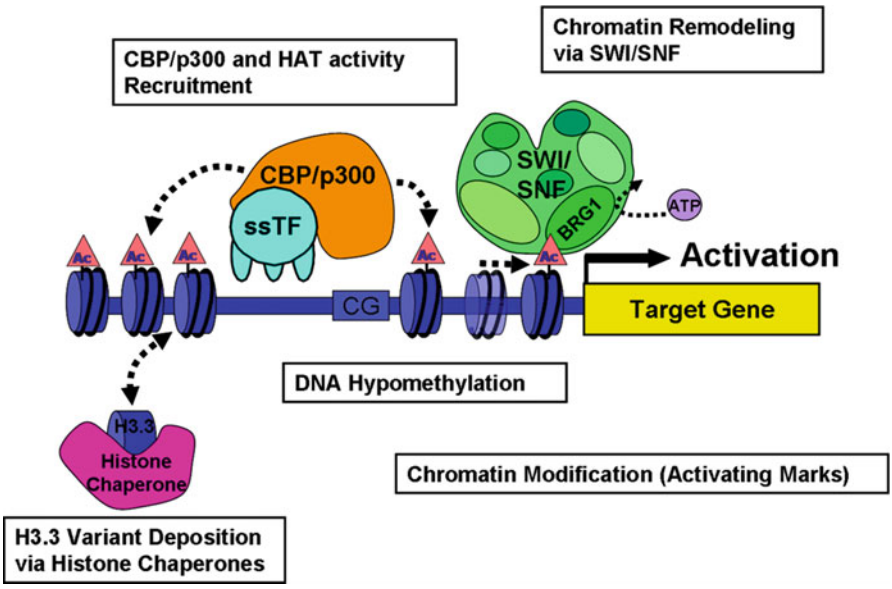

Fig. 2 Examples of Epigenetic-Mediated Oncogene Activation. This cartoon depicts a model for the role of chromatin dynamics in promoting the cancer phenotype through oncogene activation. In this model, a sequence-specific transcription factor (ssTF) triggers the recruitment of CBP/p300 (or PCAF) to a target gene promoter. The recruitment of CBP/p300 to the promoter also provides HAT activity, which facilitates the modification of surrounding histones to create "active" chromatin with acetylated histones. Addition of acetylated marks to histones signals activation of transcription through recruitment of other bromodomain-containing proteins, such as the SWI/SNF family of chromatin mechanochemical remodelers, which via the expenditure of ATP facilitate structural relaxation of chromatin and thus, access to transcriptional machinery. Additional players in the process of gene activation can include the histone chaperones, which through the exchange of histone variants, such as histone H3.3, provide activating signals. In addition, demethylation of DNA can trigger the activation of an oncogene promoter

The Histone Code and Subcode Hypotheses: Codifying Gene Activation and/or Silencing and Epigenetics

Elegant work from many laboratories around the world found its conceptual integration in the development of the histone code hypothesis [26]. Before describing this theoretical framework for understanding transcription and epigenetics, one should remember that histones are small, basic proteins that are extremely conserved throughout evolution [27]. To illustrate how conserved histones are and better explain how the histone code hypothesis operates, histone H3 (H3) is used here as an example, although the code considers all the histones and its genetic variants.

The first 24 amino acids of H3 are nearly identical in most organisms, known as the histone H3 tail. Collectively, the histone "tails" have been defined, from analysis of their crystal structure, as the regions of the histone sequences that extend from the nucleosomal disk [28]. The H3 tail contains several serine(S), threonine(T), and tyrosine(Y) residues, which have the ability to undergo phosphorylation, and other

residues, such as lysine(K) and arginine(R), which can be extensively modified by methylation, acetylation, ubiquitination, and sumoylation [26]. In fact, the lysines and arginines have the potential to possess different states of methylation, namely mono-, di-, and tri-methylated for lysines and mono-, symmetrically di-, and asymmetrically di-methylated for arginines [29]. These histone modifications have come to be known as "marks" because in many cases, they are utilized as clues for epigenetics. For instance, the Polycomb complex, which keeps stem cells in their undifferentiated state, binds to trimethylated K27 of H3 in order to mediate heterochromatin formation on target promoters and, as consequence of this event, to facilitate gene silencing [30]. This is one of the mechanisms for epigenetic inheritance in human somatic cells where the K27 trimethyl mark must be removed to initiate the hierarchical cascade of gene expression that leads to a cell fate decision. Interestingly, as described below, this epigenetic mechanism is often used for permanently silencing tumor suppressors without the need of gene mutation or deletion (Fig. 1). A similar function in gene silencing is performed by another protein, HP1, which binds to di- and tri-methylated K9 of H3. The histone code hypothesis predicts that the type, location, and combination of histone marks determine whether a gene is expressed or silent under a particular set of circumstances. Using HP1 as a model of a histone mark-binding protein, these nonhistone proteins were found to also be modified by the same enzymes that are responsible of creating the histone code, appearing to act in the fine-tuning of the instructions given by the histone marks [31], which has been subsequently supported by additional modifications in HP1 and other epigenetic regulators [32–34]. For instance, a required step for entering into cell senescence is the phosphorylation of HP1γ at residue S83 (S93 from alternative start site) [35], suggesting that this modification instructs HP1 to regulate the gene expression of key genes which will epigenetically influence the cell into senescence. In fact, the underlying mechanism driving these subcodes is believed to be "histone mimicry," which is the presence of histone-like modification cassettes within nonhistone proteins [36]. Thus, the histone code and its subcodes have fueled a new era of great productivity and optimism in the field of transcription, chromatin dynamics, and epigenetics, in particular as it relates to cancer.

Nucleosome Remodeling Machines

Nucleosome remodeling machines, containing ATP-dependent mechanochemical activity (molecular motors), were discovered using biochemical methods and in vitro assays. Using these approaches, numerous laboratories have isolated protein complexes that move nucleosomes along DNA thereby removing a repressive effect of histones on a specific cis-regulatory sequence. These nucleosome remodeling complexes include SWI/SNF, NuRD (nucleosome remodeling and deacetylation), and CHRAC (chromatin accessibility complex) (reviewed in [37]). Several of these molecular machines are conserved from organisms ranging from yeast to human. To demonstrate the basic mechanisms of these nucleosome remodelers, the SWI/SNF complex will be used as an example, which is the human homolog to the *Drosophila* trithorax complex [38]. The function of complexes like SWI/SNF is essential for the

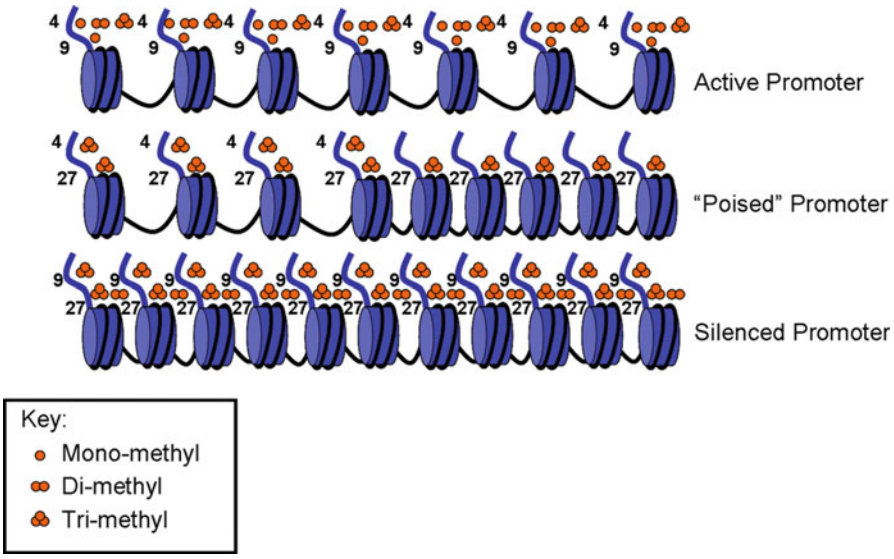

Fig. 3 Dynamics of Chromatin Marks on Promoters. The figure demonstrates three different promoter states of chromatin marks: active, "poised," and silenced (adapted from [44]). Nucleosomes encompassing the promoter region of a gene are shown. The numbers indicate the corresponding amino acid of the histone H3 tail. The orange circles represent the degree of methylation with multiple states possible for a given signal. For example, on active promoters, the chromatin marks are a signal of gene transcription, such as mono-, di-, or tri-methylation of K4 of H3 and mono-methylation of H3K9. Active promoters are also enriched in H3, H4, and H2A acetylation (not shown). On a "poised" promoter, a combination of active and repressive marks can leave genes ready for activation and forms a "bivalent domain." The promoter regions of this type are enriched in the repressive trimethyl-H3K27 mark, whereas the region around the transcription start is also enriched in the active trimethyl-H3K4 mark. Finally, a silenced promoter contains inactive chromatin marks. These nucleosomes are enriched in H3K9 tri-methylation (and sometimes di-methylation, not shown) and H3K27 di- and tri-methylation

expression of a myriad of genes via its recruitment to chromatin, hydrolysis of ATP, and utilization of this energy to remodel nucleosomes (Fig. 2). While *Drosophila* only possesses a single Swi2/Snf2 complex with ATPase activity, called Brahma (Brm) [39], mammals have two homologues, BRM and BRG1 [40]. The amino acid sequences of these two are 75% identical with broad expression. However, these subunits are mutually exclusive, since a single SWI/SNF complex contains either BRM or BRG1. Thus, there are several subtypes of SWI/SNF complexes that can be divided based on the ATPase molecule that generates the mechanochemical force for nucleosome movement. Interestingly, the genes encoding these subunits have been found to have mutations and/or loss of expression in some human tumor cell lines, as well as primary tumors, including pancreatic cancer [41, 42].

The trithorax complex recognizes methylated H3K4, actively participating in the epigenetics and chromatin dynamics of the cell. For instance, stem cells are characterized by having a subset of genes with dual marks, methylated at both H3K4 and

H3K27 (Fig. 3). These gene promoters are known to be in a "poised" state, since they are repressed by polycomb in the stem cells, but after removal of the dominant H3K27 mark, the remaining methylated H3K4 will signal for activation, leading to the initiation of cell differentiation [43]. Therefore, although heterochromatin is repressive, nucleosome remodeling machines, by binding to specific histone marks, sometimes already present on a promoter along with the silencing mark, will convert the region into active euchromatin. Tumorigenesis exhibits the culmination of alterations in several genetic pathways. Therefore, as is the case with many of the global epigenetic effects discussed in this chapter, it would only take a single mutation to inactivate a large subset of SWI/SNF complexes (such as a BRG1 mutation) to perturb the regulation of numerous downstream genetic pathways and as a result, trigger robust growth-promoting effects (Fig. 2).

Histone Chaperones

The discovery of histone chaperones constitutes later developments within the area of transcription [44]. The search for this type of proteins initiated from the understanding that there were many histones and histone variants that could occupy a nucleosome. For instance, histone H3 has four main isoforms in mammals [45]. Some of these variants act as activators, while others act as repressors in the context of a nucleosome [46]. Deposition of histone variant H3.3 has been associated with transcriptionally active genes in plants, flies, and humans. In addition to the possibility of different histone variants occupying a nucleosome, these variants are also substrates of enzymes that create histone marks. Therefore, the combinatorial effect between the existence of the histone variants and their participation in the histone code, which is known as the histone "barcode" [47], creates the possibilities of regulating activation or repression significantly complex. An important contribution to the field was the discovery that some histone variants are rapidly exchanged from nucleosomes, leading to the finding that this nucleosome-histone exchange codifies for either gene activation or silencing. Therefore, histone chaperones cooperate with the histone code in instructing cells to regulate a particular program of gene expression (Fig. 2). The role of histone chaperones involves binding highly basic histone proteins, which protects them from nonspecific interactions to facilitate either their deposition onto or eviction from DNA. Interestingly, despite their common functions, histone chaperone proteins structurally demonstrate highly divergent molecular structures and modest commonalities in their folds [47]. However, according to sequence-based predictions, these proteins have recently been shown to contain critical intrinsically disordered regions (IDRs) and acidic stretches, which are thought to play key roles in histone chaperone function, although this remains a currently active area of research.

Nuclear Shape and Nuclear Domains

The influence of nuclear shape in determining the tridimensional location of a particular gene within the nucleus in interphase is well known (chromosome

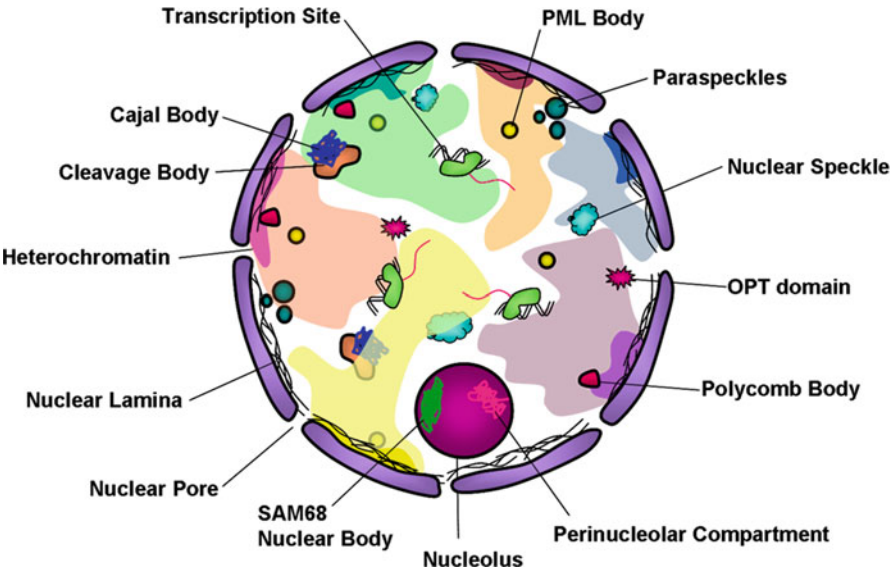

Fig. 4 Chromosomal Territories and Nuclear Domains. This cartoon of a mammalian nucleus illustrates the chromosomal territories and various nuclear bodies. Chromosomes occupy discrete territories in the nucleus. In addition, various functions within the nucleus occur in distinct locations, considered nuclear bodies or domains. Recent important and elegant work has demonstrated that alterations in nuclear shape will impact on these nuclear territories and domains, affecting gene expression in a manner resembles aging, polyploidy, and aneuploidy, all changes that are found in pancreatic cancer. Therefore, extending this area of research is of paramount importance for this field

territory) [48]. In addition, the nucleus consists of distinct nuclear domains with various components, which suggests that various nuclear functions occur at precise locations within the nucleus (Fig. 4). This knowledge supports the notion that changes in nuclear shape, by altering the nuclear position of the gene, can alter chromatin dynamics leading to aberrant gene expression. Clear support for this concept came from a naturally occurring mutation in the Lamin A gene [49]. Lamins are proteins that form intermediate filaments, which create a nuclear lamina covering the nucleus and extend toward the interior of this organelle to form a skeleton (reviewed in [50]). Thermodynamically speaking, the efficiency of an enzyme is better when in association with a surface rather than free floating in solution. Therefore, this lamin-based skeleton is necessary for all the processes that occur in the nucleus by helping to compartmentalize and concentrate specific molecular machineries into nuclear domains, which can be considered the nuclear equivalent of the cytoplasmic organelle, though not surrounded by a membrane. Mutations in lamin A significantly change nuclear shape, generating a new pattern of gene expression, which is responsible for the phenotype of premature aging and cancer in the Hutchinson–Gilford progeria syndrome [49]. With increasing focus on the functional relevance of morphological changes in the size and shape of the nucleus

during tumorigenesis, studies have found both increased and decreased lamin A/C levels to be correlated with poor prognosis in human cancers [51]. Notably, in considering the critical role of the tumor microenvironment in pancreatic cancer, aberrant levels of lamin A/C are also associated to collagen deposition and fibrosis, suggesting its effect reaches beyond the nuclear structure to influence the tissue architecture and microenvironment. This has inspired our laboratory to predict that some of the gross nuclear changes observed early during the progression of histopathological lesions in pancreatic cancer are not a consequence of cancer, but rather these changes help in the development and/or maintenance of this malignant phenotype. Therefore, nuclear shape must be included as a candidate modifier of pancreatic cancer progression, since the transition of PanIN 1B to PanIN 2 requires changes in nuclear shape [52]. The hypothesis is that these nuclear changes are responsible for extensively altering gene expression, independently of other epigenetic mechanisms, and thereby significantly contribute to the progression and maintenance of the pancreatic cancer phenotype. Thus, the "Triple Code Hypothesis," as illustrated in Fig.5a, is an integration of changes in DNA, such as mutation or deletion, which are an established part of cancer progression, alterations in chromatin, which are increasingly recognized as well, and the addition layer of changes in nuclear structure [53].

Epigenetics: Developing a Novel and Comprehensive Genomic-Epigenomic Model for Pancreatic Cancer that Includes Chromatin Dynamics and Nuclear Shape

The revolution of somatic genetics in the field of cancer brought about by the model developed by Fearon and Vogelstein in colon [54], which later led to an adaptation to the pancreas by Hruban et al. [1], opened a fruitful era for pancreatic cancer research, spanning approximately two decades. The basic premise of somatic genetics in cancer is that if a gene, which is suspected to play a role related to cancer, is overamplified, for instance, Myc in brain, it behaves as an oncogene, but if it is downregulated, like p16 in pancreatic cancer, it behaves as a tumor suppressor. Due to this premise, in the pancreatic cancer field, the changes in expression of both oncogenes and tumor suppressors, according to the Hruban model, were originally believed to occur via mutation or deletion and later with the work of Goggins, by promoter methylation [55–57]. The validity of this model has been elegantly demonstrated using Genetically Engineered Models (GEM), primarily supported by NIH via the "Mouse Model Consortium" funded by NCI [58].

In addition to the recognition of the outstanding contribution, this progression model of somatic genetics has had in advancing cancer research, the revised progression model for pancreatic cancer also must take into consideration the theoretical framework of epigenetics, and specifically, changes that occur at the protein level in the absence of DNA changes, such as deletion, mutation, or even promoter methylation. For instance, upon reading through the Hruban model of pancreatic cancer, in which the underlying conceptual framework is genetic in nature, one can infer that

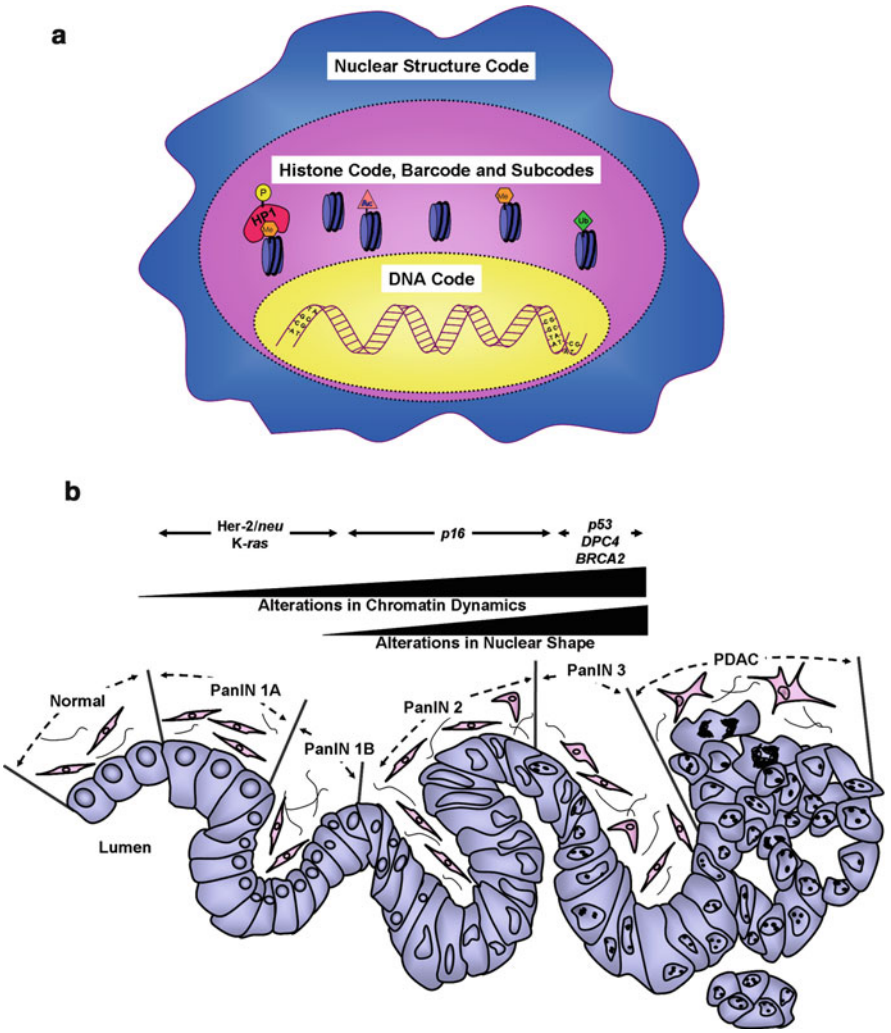

Fig. 5 (**a**) **The Triple Code Hypothesis**. This figure summarizes the integration of the well-known DNA-centric hypothesis for the establishment and maintenance of the cancer phenotype, which includes mutations and deletions, with changes in chromatin, signaled through the histone code, barcode, and its subcodes, and alterations in nuclear structure to form the "Triple Code Hypothesis." This "Triple Code Hypothesis" has formed the basis of the more comprehensive progression model for pancreatic cancer, proposed in **b**. (**b**) **Revised Comprehensive Progression Model for Pancreatic Cancer**. The model developed by Hruban and colleagues [1] was fundamental for expanding the work of many laboratories in the area of somatic genetics in pancreatic cancer to allow better understanding of the relationship between the morphological progression and mutations/deletion of important oncogenes and tumor suppressor pathways. However, the model excludes emerging knowledge on critical steps that occur between these mutations and even the potential cause of subsequent mutations and deletions. Most of these changes are epigenetic in nature with the underlying basic mechanisms of both chromatin dynamics and nuclear shape. Thus, a revised model for the progression of pancreatic cancer [53], which not only incorporates the

pancreatic cancer progresses through multistep mechanisms with different lesions evolving via mutations in different genes. However, this model does not explain what protein-mediated epigenetic changes, which can take place between the occurrences of landmark mutations, are responsible for cancer progression, nor this model has proven that a later mutation is caused by an earlier one. Therefore, in the following paragraphs, examples of epigenetic changes that occur in time between mutations and can lead to tumor suppressor silencing are provided, starting with DNA methylation and proceeding through some modifiers of chromatin. These examples highlight a paradigm for the progression of pancreatic cancer, which includes two additional types of phenomena (besides genetics), namely changes in chromatin dynamics and nuclear shape (Fig. 5b). The hope is for new investigators in this field to dive into pancreatic cancer with a more in depth mechanistic approach than using only the tools of molecular pathology and a combination of a multitude of arrays for different purposes.

While the field of epigenetics is vast and includes mechanisms of gene activation and repression, this chapter will focus on changes in epigenetics and chromatin dynamics that can silence tumor suppressor genes via mechanisms that are totally independent of either genetic deletions or mutations. In fact, in the case of *p16*, which is utilized here as a prime example for pancreatic cancer in the following paragraphs, epigenetic mechanisms lead to the final methylation of this gene, which should take the readers to consider that chromatin changes can occur before and lead to the inactivation of landmark mutations that were described in the original paradigm. Therefore, this journey will begin with a brief description of this final read-out in epigenetics, DNA methylation, since it is the most commonly known epigenetic alteration, and continue temporally backwards in epigenetics toward changes in chromatin and their modifiers. In addition, studies in the epigenetics of noncoding RNAs in pancreatic cancer will be described, which is the most recent area to develop in the field.

DNA Methylation

As mentioned, DNA methylation was the first type of epigenetic change to be studied as a mechanism for the inactivation of tumor suppressors [59]. DNA methylation occurs on dinucleotide CpGs, where cytosines precede guanines. The process of DNA methylation entails the addition of a methyl group to the number 5 carbon of

Fig. 5 (continued) elegant and extremely important data generated under the premise of the original model but, in addition, formally includes chromatin-induced and noncoding RNA-induced epigenetic changes, as well as other alterations caused by changes in nuclear shape, is illustrated. This model will hopefully serve as a compass to guide future experiments in these underexplored and yet crucial areas of knowledge. Experiments aimed at addressing the contribution of these phenomena to pancreatic cancer progression and their potential translation to clinical applications will be among the most promising areas of our field

the cytosine pyrimidine ring, which ultimately silences gene expression. Noteworthy, DNA methylation normally has significant physiological significance, as with genomic imprinting to ensure monoallelic expression and hypermethylation of repetitive genomic sequences to prevent chromosomal instability, translocations, and gene disruption caused by the reactivation of transposable DNA sequences. However, during tumorigenesis, aberrant DNA methylation can assist the cancer phenotype.

In pancreatic cancer, DNA methylation has been known for a long time as a mechanism to inactivate tumor suppressor genes, such as well-known inactivation of the p16 promoter via methylation [60]. In addition, loss of methylation of a normally silenced promoter in pancreatic cells, such as the gene encoding the hematopoietic-specific guanine nucleotide exchange factor, *VAV1*, can lead to its misexpression [61]. Initial methodologies only provided insights at the single gene level, but fortunately, recent developments in methodologies have advanced enough to perform genome-wide scale gene methylation analysis. With validity to both methodologies, methylation analysis of a single gene is practical as a specific candidate gene approach, while the genome-wide analysis possesses power in its unbiased approach. Several techniques utilized for methylation analysis include methylation-specific PCR, sequencing after bisulfite treatment, as well as mass spectrometry.

Although individual genes were discovered to be methylated in advanced pancreatic cancer, current evidence supports the idea that aberrant methylation occurs very early during the histopathological progression of this neoplasia. Using a specific gene candidate approach, Rosty and colleagues demonstrated that PanIN lesions in patients with chronic pancreatitis show loss of *p16* expression [62], suggesting that this alteration may contribute to the predisposition of patients with chronic pancreatitis to develop pancreatic ductal adenocarcinoma. Interestingly, in a study involving large-scale methylation analysis with subsequent confirmation via methylation-specific PCR, Sato and colleagues analyzed DNA samples from 65 PanIN lesions for methylation status of eight genes identified prior by a larger scale microarray approach as aberrantly hypermethylated in invasive pancreatic cancer [63]. Of the PanIN lesions examined in this study, methylation at any of these genes was identified in 68% of samples. Even more importantly, in the earliest lesions, which are the PanIN-1A, aberrant methylation was present in approximately 70%. Among the genes analyzed, methylation prevalence increased from PanIN-1 to PanIN-2 for *NPTX2* and from PanIN-2 to PanIN-3 for *SARP2*, *Reprimo*, and *LHX1*. The most striking result from both studies is that aberrant CpG island hypermethylation begins in early stages of PanINs and its prevalence progressively increases during neoplastic progression.

Additional studies on methylation patterns in pancreatic cancer compared to nontumor pancreatic tissues have followed to demonstrate a high level of differently methylated regions (DMRs) between the two groups, which offer a large list of candidate genes to serve as diagnostic biomarkers or therapeutic targets [64, 65]. A more recent study, using reduced-representation bisulfite DNA sequencing (RRBS) followed by targeted methylation-specific PCR to validate novel DNA methylation markers strongly associated with pancreatic cancer, could discriminate pancreatic

cases from controls in pancreatic juice, which offers clinical significance in terms of detection and would benefit further validation in patients with early PanIN lesions [66]. With the current interest in circulating cell-free DNA (cfDNA), Henriksen and colleagues identified differences in cfDNA promoter hypermethylation between malignant and benign pancreatic disease to suggest its utility as a noninvasive, blood-based screening tool for pancreatic cancer [67]. Thus, aberrant DNA methylation not only continues to reconfirm its clear role in the progression of pancreatic cancer, but holds promise as a diagnostic marker. Furthermore, since the current evidence indicates that methylation occurs at an early preneoplastic stage, pharmacological agents that target methylation, which are discussed in a subsequent chapter on "Epigenetic Pharmacology," may be effective not only for treatment, but perhaps also for chemoprevention.

Histone Acetylation and Deacetylation

An important mechanism underlying the epigenetic regulation of gene expression is the acetylation and deacetylation of lysine residues within histone tails [68]. For acetylation, this process occurs via HATs, such as CBP, P300, and PCAF, to result in gene expression activation, whereas deacetylation is mediated by two different families of HDACs, resulting in gene silencing. Together, these enzymes provide a fine-tuned mechanism, which upon alteration has the possibility to cause the activation of oncogenic pathways (Fig. 2) and the silencing of tumor suppressors (Fig. 1). However, apart from other epigenetic regulators, such as the polycomb complexes and HP1, which are discussed below, HATs and HDACs mediate short-term responses, a fact that should be taken into consideration when thinking about these molecules as potential therapeutic targets in cancer [68, 69].

As discussed, transcriptional regulation is mediated by the DNA binding properties of sequence-specific transcription factors and the recruitment of trans-activators or repressors to ultimately cause effects that alter chromatin structure and dynamics. Studies have demonstrated that HDAC activity is increased in various tumors compared with normal tissue, and this increase in HDAC activity has been associated with transcriptional repression of tumor suppressor genes that cause growth inhibition and apoptosis [70]. In a study performed by Blasco and colleagues, the differential gene expression in a pancreatic cancer cell line upon induction of apoptosis was analyzed using cDNA arrays [71]. Among the genes differentially expressed, one that was studied for further validation was histone deacetylase 1 (HDAC1). Inhibition of HDAC activity led to an increase in the level of apoptosis, in parental cells and doxorubicin-resistant cells. Thus, this study suggested that HDAC1 could be a possible target to develop modulators in cancer chemotherapy that would increase or restore apoptosis. In another study performed by Ouaïssi et al., approximately 80% of pancreatic adenocarcinoma samples examined showed a significant increase of HDAC7 RNA and protein levels [72]. Interestingly, in contrast to the pancreatic adenocarcinoma samples, HDAC7 RNA levels were reduced in samples from chronic pancreatitis, serous cystadenoma, and intraductal

papillary mucinous tumor of the pancreas (IMPN), suggesting that increased expression of HDAC7 can discriminate pancreatic adenocarcinoma from other pancreatic types of tumors. Immunohistochemical assessment of HDAC1, HDAC2, HDAC4, and HDAC6 protein levels in 70 PDAC patient tissue samples demonstrated enhanced HDAC1 levels in association with increased tumor proliferative capacity, while elevated HDAC4 expression was significantly correlated with the absence of organ metastases [73]. Significantly longer survival times were noted in patients with high HDAC1 and HDAC6 levels compared to those with low expression of these molecules, whereas HDAC2 had no significant association with any of the clinicopathological parameters considered. In addition, it has been shown that HDAC1 mediates transcriptional repression of the TGFβRII promoter in pancreatic ductal adenocarcinoma cells via recruitment to a specific Sp1 site [74]. This Sp1 site can be occupied by TGFβ-inducible members of the KLF family, including KLF14 [75] and the pancreatic tumor suppressor, KLF11 [76]. Interestingly, a genome-wide association study (GWAS) from 7683 patients with pancreatic cancer and 14,397 controls found that one of the four identified SNPs to reach genome-wide significance was located near KLF14 [77].

Using the *Pdx1*-Cre/*Kras*$^{LSL\text{-}G12D}$ mouse model of PDAC precursor lesions in combination with cigarette smoke exposure, Edderkaoui and colleagues determined that inhibition of HDAC3 reverses the accelerated PanIN formation observed from smoking and thus is a major player in mediating the pro-cancer effects resulting from this exposure [78]. This effect is facilitated, at least in part, through HDAC3-mediated regulation of IL-6 production in cancer cells to influence macrophage function, specifically the pro-tumor type-2 macrophage (M2) phenotype, in the tumor microenvironment. Several HDAC inhibitors have FDA approval, including Vorinostat, Romidepsin, and Belinostat [79], and thus, most ongoing studies in the field are focused on their use as targeted epigenetic therapeutics in PDAC, which is the topic of a subsequent chapter dedicated to "Epigenetic Pharmacology." In summary, it is clear that HDACs play an important role in the maintenance of the proper balance of chromatin marks on a given promoter, and if this balance is altered, such as HDAC expression in pancreatic cancer, the expected global effect on promoters is daunting.

Histone H3-Methyl-K27 and Polycomb

Polycomb proteins silence gene expression by specific methylation of histone H3 on K27 [68, 80]. At the simple core of this pathway, polycomb group (PcG) proteins act via the stepwise recruitment of PRC2, containing the H3K27 methylase activity, to chromatin. Subsequently, the trimethyl-H3K27 mark deposited by PRC2 recruits the PRC1 complex, thereby completing the gene silencing complex formation. The enzymatic activity of the PCR2 complex involves the H3K27 histone methylase, EZH2, but requires a complex with Suz12 and EED to function. The PCR1 complex contains the oncogene BMI1, as well as HPC1–3, HPH1–3, SCMH1, and the methyl-H3K27-binding proteins, Cbx 2, 4, 6, 7, and 8. However, which of the Cbx proteins is active at different loci under different circumstances is not known.

The role of polycomb proteins in pancreatic cancer has elicited significant attention over the recent years. For instance, new polycomb proteins have been discovered in pancreatic cancer cells [81]. More importantly, studies have demonstrated that loss of trimethylation at H3K27, which is achieved by EZH2, is a predictor of poor outcome in pancreatic cancers [82]. In fact, together with tumor size and lymph node status, the level of trimethyl-H3K27 was found to have a strong and independent prognostic influence in pancreatic cancer. Nuclear accumulation of EZH2 was identified as a hallmark of poorly differentiated pancreatic adenocarcinoma, and this nuclear overexpression of EZH2 contributes to pancreatic cancer cell proliferation, suggesting EZH2 as a potential therapeutic target for the treatment of pancreatic cancer [83]. In samples obtained by endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA), EZH2 expression was determined by immunohistochemistry to evaluate its use as a potential biomarker for treatment and disease prognosis [84]. However, EZH2 expression was heterogeneous and did not correlate inversely with E-cadherin expression as expected to serve as a hallmark of poorly differentiated pancreatic adenocarcinoma. Nevertheless, interest remains high for EZH2 as a therapeutic target in PDAC. Using the cerulein-induced model of pancreatic injury, EZH2 levels increase after injury, and this methyltransferase is required to promote the tissue repair process through inducing regenerative proliferation of progenitor cells [85]. With genetically engineered animal models, the same study revealed that EZH2 knockout impairs pancreatic regeneration and accelerates $KRas^{G12D}$-driven PanIN formation. Recent investigations found that activated CDK5 kinase is responsible for EZH2 phosphorylation, which is required for F-box and WD repeat domain-containing 7 (FBW7) to target EZH2 for ubiquitination and subsequent degradation [86]. As a result, this process suppresses EZH2 activity and thereby inhibits tumor migration and invasion of pancreatic cancer cells, not only highlighting the role of EZH2 overexpression present in PDAC samples, but providing additional therapeutic targets as well.

In terms of the PRC1 complex, a study on the ubiquitin E3 ligase Ring1B, a key component of PRC1 by catalyzing monoubiquitination of histone H2A at lysine 119 (H2AK119Ub1), and Snail, a transcriptional repressor and master regulator of epithelial-mesenchymal transition (EMT), demonstrated that elevated levels of these two molecules along with elevated monoubiquitination of H2AK119 are highly correlated with poor survival in PDAC [87]. On the other hand, reduction in CBX7 levels was associated with increasing malignancy grade in pancreatic adenocarcinoma and correlated with a loss of E-cadherin expression [88]. Conservation of CBX7 levels trended with longer patient survival rates, suggesting that loss of this polycomb protein contributes to a more aggressive pancreatic cancer phenotype. Moreover, CBX7 plays a role in suppression of cell proliferation, migration, and invasion, which is thought to occur in part through reducing PTEN/Akt signaling [89]. Pancreatic cancer stem cells, a small subset of distinct cancer cells with great proliferative potential and resistance to standard therapies, were identified to have upregulation of the PRC1 molecule Bmi-1, which enhances tumorigenicity and the function of the cancer stem cell population [90]. Interestingly, similar to CBX7, Bmi-1 influences the Akt signaling pathway, but by activating PI3K/AKT signaling

through the negative regulation of PTEN [91]. This mechanism was found to stimulate invasion and metastasis of the pancreatic cancer stem cells. Pancreas-specific inactivation of Bmi-1 in the *Pdx1*-Cre/*Kras*$^{LSL\text{-}G12D}$ murine model of pancreatic cancer initiation suggested that Bmi-1 is required for this process, in an Ink4a/Arf-independent manner [92]. Loss of Bmi-1 resulted in the upregulation of ROS, indicating that this PRC1 molecule regulates protection from excess ROS during neoplastic transformation, which is required for survival and progression. Thus, the association of this pathway with poor survival of patients affected by this disease renders this area of research one of paramount importance.

Mechanistically, one of the outcomes of aberrant polycomb regulation is the silencing of the *p16* gene, which could occur prior to DNA methylation, via altered direct recruitment of members of this family to the *p16* promoter sequence [93]. Upon studies in human cells, EZH2 and DNA methyltransferases (DNMTs) were found to physically and functionally interact, evidenced by the PRC2 subunits, EZH2 and EED, co-immunoprecipitating with all three human DNMTs and the co-dependency of certain target gene silencing requiring both EZH2 and DNMTs [94]. Therefore, the presence of polycomb proteins on the *p16* promoter can recruit DNA methylases which then further inactivate the expression of *p16* via DNA methylation (Fig. 1). However, whether histone H3K27 methylation and recruitment of DNMT to result in DNA methylation ultimately leads to permanent mutation/deletion of the gene or all mechanisms of *p16* inactivation are independent remains to be discovered.

Histone H3-Methyl-K9 and Heterochromatin Protein 1

As described in a prior section, HP1 binds methylated K9 of histone H3, causing transcriptional repression [68, 95]. This occurs through the N-terminal chromodomain of HP1, while the highly related C-terminal chromoshadow domain allows for dimerization of these HP1 molecules and serves as a docking site for various factors involved in a wide array of functions, from transcription to nuclear architecture. To mediate gene silencing via the formation of heterochromatin, HP1 isoforms must interact with different H3K9 histone methylases, G9a (EHMT-2), GLP (EHMT-1), and SUV39H1 [68, 95]. These methylases work in concert with HP1 in a circular manner to form silenced chromatin. When the methylases adds methyl groups to K9 of H3, this, in turn, forms an HP1 docking site on chromatin. Since HP1 also recruits the methylases, this cycle repeats, and the HP1–methylase pair can spread the formation of silenced chromatin to adjacent nucleosomes, causing long-term silencing of entire genes (Fig. 1).

Information regarding the function of HP1 proteins in both normal and tumor pancreatic cells is still emerging. However, HP1 proteins have altered expression in many different types of cancers, including breast, brain, ovarian, colon, and papillary thyroid cancers as well as leukemias [96]. Noteworthy, with the three human isoforms having over 80% similarity between them, the factors that influence these

differences remain unknown. Unfortunately, despite the identification of numerous HP1 binding partners, distinct signaling cascades that mediate the interaction with these proteins to ultimately "switch on" or "switch off" gene silencing remain largely unknown. Although the discovery of the previously mentioned HP1-mediated subcode [31] contributed to this understanding, it remains essential to carefully define these pathways to map useful networks of membrane-to-chromatin signaling cascades for better understanding of the regulation of activation, repression, as well as other cellular processes. The molecular mechanisms that operate as subcodes within the histone code trigger nuclear instructions imparted by H3K9 methylation, which are subsequently translated as silencing, and thus, potentially participating in the silencing of tumor suppressor genes.

One specific example of how the methyl-H3K9/HP1 type of chromatin dynamics can impact on the field of pancreatic cancer is the regulation of MUC1 expression. The sialylated form of MUC1 is overexpressed in invading and metastatic pancreatic cancer cells, but absent in normal pancreas, cases of chronic pancreatitis, and pancreatic ductal hyperplasia [97], lending this molecule to be an interesting target for immunotherapeutic strategies [98]. Strikingly, studies have recently demonstrated that a mechanism responsible for changes in the expression of MUC1, which can in turn make proposed vaccines less than optimal, is regulated by DNA methylation and H3K9 modification, which is bound by HP1, on the *MUC1* promoter [99]. Similar to polycomb, it is known that HP1 can recruit DNA methyltransferases [100], which can lead to the silencing of this important molecule for pancreatic cancer (Fig. 1). MUC1-negative cancer cell lines correlated with high DNA methylation and methyl-H3K9 levels, while MUC1-positive cell lines had low levels of these epigenetic marks. Increased expression of NFATc2 in advanced PanIN-2/PanIN-3 lesions and PDAC coincides with silencing of the $p15^{INK4b}$ tumor suppressor pathway, which mechanistically has been linked to recruitment of SUV39H1, to result in H3K9 trimethylation and subsequent binding of HP1γ [101]. Interestingly, the first genome-wide study on the epigenetic landscape, comparing matched primary and metastatic PDAC lesions collected by rapid autopsy, revealed widespread epigenetic reprogramming during the evolution of distant metastasis without the presence of metastasis-specific driver mutations [102]. This reprogramming presented as global changes specifically in histone H3K9 and DNA methylation within large heterochromatin domains, known as LOCKs, as well as regional changes in histone marks, such as acetyl-H3K27 at gene regulatory elements. Inhibition of the H3K9 pathway results in senescence of pancreatic cancer cells without inducing apoptosis, thereby reducing anchorage-dependent and anchorage-independent proliferation [103]. Furthermore, the combined inhibition of the Aurora kinase A oncogene with the H3K9 pathway impedes PDAC cell growth via a mechanism that, instead of senescence, involves perturbation of normal mitotic progression to end in mitotic catastrophe [104]. Therefore, chromatin dynamic-driven epigenetic changes have the potential to extend research beyond the minimal mutation paradigm to include other pathways that are also important for other key biological behaviors in pancreatic cancer.

Additional Nonhistone Chromatin Proteins as Epigenetic Targets

Other nonhistone chromatin proteins, such as the Sin3a scaffold, play a role in pancreatic cancer [105]. For instance, pancreatic cells express three different Sin3 proteins that are recruited by tumor suppressors, such as the Myc antagonist, Mad1, and KLF11, and these tumor suppressor proteins require binding to a Sin3a–HDAC complex to perform their function (Fig. 1). Thus, this system is both active and important for antagonizing pancreatic carcinogenesis. Furthermore, pathogenic mutations and structural variants have been discovered in several epigenetic regulator genes, resulting from whole genomic sequencing of 100 pancreatic cancer samples, including *KDM6A*, *ARID1A*, *ARID1B*, *PBRM1*, *SMARCA2*, *SMARCA4*, and *MLL2* [106]. Interestingly, *KDM6A*, which encodes for an H3K27me3 demethylase, was inactivated in as much as 18% of the pancreatic cancer patients. Another KDM6 family member, KDM6B, which also demethylates H3K27me3, has loss of heterozygosity in pancreatic cancer cells and its loss is associated with enhanced tumor sphere formation, as well as increased peritoneal dissemination and liver metastasis in vivo [107]. Thus, the future anticipates studies of these various complexes in the context of pancreatic cancer, which may reveal significant contributions to the initiation, maintenance, or spreading of this disease or to cancer-associated functions, such as stem cell maintenance, DNA repair, metastasis, and therapeutic response.

Noncoding RNAs and Pancreatic Cancer

Due to the discovery and increasing study of noncoding RNAs, including microRNAs (miRNAs), short interfering RNAs (siRNAs), piwi-interacting RNAs (piRNAs), and long noncoding RNAs (lncRNAs), a significant number of researchers are analyzing noncoding RNA signatures in pancreatic cancer. The best-characterized noncoding RNAs are miRNAs, which are endogenous noncoding RNA molecules approximately 21 nucleotides in length that have been found to play important roles in the regulation of genes in animals and plants via a process involving their pairing to the mRNAs of protein-coding genes to direct their post-transcriptional repression [108]. In fact, miRNAs are currently predicted to control the activity of approximately 30% of all protein-coding genes in mammals. Similar to coding transcripts, miRNAs are classified into oncogenic miRNAs and tumor suppressor miRNAs in relation to their function during tumorigenesis. In an early global profiling study, several miRNAs were identified as aberrantly expressed in pancreatic cancer or desmoplasia [109]. Interestingly, some of these have been previously reported as differentially expressed miRNAs in other human cancers, including miR-155, miR-21, miR-221, and miR-222, in addition to some novel ones not previously reported, such as miR-376a and miR-301. Typically, the most aberrantly expressed miRNAs were found to be downregulated in the tumor tissue. Several additional profiling studies have found miRNA deregulation in human PDAC. In another study, several miRNAs, including miR-205, −18a, −31, −93,

−221, and −224, were demonstrated to be overexpressed in primary neoplastic ductal cells and pancreatic cancer cell lines, representing promising biomarkers for pancreatic cancer [110]. Furthermore, 26 miRNAs were identified as the most significantly misregulated in pancreatic cancer and the analysis of only two miRNAs, miR-217 and -196a, allowed discrimination between normal and neoplastic tissues, further supporting the potential use of miRNAs for the diagnosis of pancreatic cancer. Bloomston and colleagues also performed a global analysis to compare miRNA profiles of normal pancreas, chronic pancreatitis, and pancreatic adenocarcinoma [111]. In 90% of the tested samples, 21 overexpressed and 4 downregulated miRNAs were capable of differentiating pancreatic cancer from benign pancreatic tissues via cross validation. Additionally, 15 miRNAs demonstrated increased expression and 8 showed decreased expression, which could distinguish pancreatic cancer from chronic pancreatitis with 93% accuracy. Noteworthy, a subgroup of 6 miRNAs was able to discriminate node-positive disease between long-term survivors and patients who would succumb to the disease within 24 months. Poor survival of pancreatic cancer, with a median survival of 14.3 months versus 26.5 months, could be predicted with 95% confidence through high expression of miR-196a-2.

Certainly, the studies of miRNAs in pancreatic cancer in general have grown significantly over the last decade. However, with increased interest and focus on identifying circulating biomarkers in PDAC as a noninvasive, cost-effective, and reliable means to detect and/or monitor the disease, it is important to discuss the use of miRNAs in this context, as well as the contribution of circulating miRNAs to the disease. miRNAs can be detected in human plasma, circulating as free RNAs, either bound to hAgo2 or included in exosomes, which are stable and protected from endogenous RNase activity [112]. The first relatively large study performed by multiple independent centers reported that 29 circulating miRNAs from pretreatment blood samples collected before clinical or surgical intervention had the potential to differentiate PC cases from healthy volunteers [113]. Of these, 13 miRNAs were selected for further validation. While their diagnostic value was not significantly different than CA19–9, this report represented a proof-of-principle that circulating miRNAs can serve as potential biomarkers for early pancreatic cancer. A meta-analysis performed on 29 published studies, including a total of 2225 patients and 1618 controls, to evaluate the diagnostic accuracy of circulating miRNAs for pancreatic cancer diagnosis found multiple miRNAs to have a relatively high diagnostic value compared to single miRNA diagnosis [114]. A retrospective screen of early stage pancreatic cancer patients and controls detected 15 differential candidate miRNAs in plasma samples from pancreatic cancer patients at diagnosis [115]. However, these circulating miRNAs, alone or in combination, were not significantly altered in prediagnostic plasma samples from an early detection case-control cohort, suggesting that these miRNAs emerge late in disease development and would not function for early detection. Studies of this nature are still in their relative infancy, and if reliable circulating miRNAs are identified for early detection and/or monitoring disease progression, this noninvasive and cost-effective window into an epigenetic signature has a promising future in clinical application.

In addition to miRNAs, another class of noncoding RNAs that have elicited attention as novel drivers of tumorigenesis are long noncoding RNAs (lncRNAs). lncRNAs are longer than 200 nucleotides in length with their genomic location mainly in intronic and intergenic regions [116]. These RNAs are transcribed by RNA polymerase II, even with similar mRNA structures, such as a 5′ cap and a 3′ poly (A) tail, and based on the proximity to protein-coding genes are classified as antisense, sense, bidirectional, intronic, and intergenic lncRNAs. lncRNAs are believed to function in a variety of ways, including as cis- or trans-regulators of gene activity, as scaffold elements, guides, or decoys for chromatin-modifying complexes, or as gene enhancers. In respect to pancreatic cancer, recent studies have revealed several lncRNAs with differential expression in pancreatic cancer, including well-known lncRNAs such as H19, HOTAIR, HOTTIP, and MALAT-1, among others [117]. Even though most non-protein-coding transcripts belong to this class of RNAs, representing more than 20% of the genome, their highly diverse structures and functions provide a source of much understanding that remains unknown regarding these molecules in both, health, and disease.

In summary, the revised paradigm for the better understanding and promoting further research in pancreatic cancer, besides taking into consideration only mutations and deletions, as well as promoter DNA methylation, now includes both chromatin dynamics, noncoding RNAs, and nuclear shape (Fig. 5a, b). It is noteworthy to underscore that although more work on chromatin dynamics is needed to understand pancreatic cancer development and phenotype, little has been done about the role of nuclear shape in this disease. Therefore, the purpose of this model is to further fuel a new era of experiments that expand the scope of the field from a DNA-centric paradigm to a holistic and more inclusive model, which takes into consideration protein-mediated epigenetics, noncoding RNA-mediated effects, and the biology of the nucleus as an altered organelle in the progression of pancreatic tumors (Fig. 5a, b).

Conclusion

Increasing studies on chromatin dynamics are unveiling the existence of robust machineries that can mediate epigenetic changes in pancreatic cells. The research community needs to focus not only on somatic genetics, since this mechanism certainly does not represent the full story of alterations in gene expression for pancreatic cancer. This important fact has led to the design of a more comprehensive model that widely includes the emerging data in the field of chromatin dynamics and nuclear shape. Guided by this model, the knowledge gathered on this disease can be more accurately mapped to a progression paradigm that will not doubt impact on many areas of pancreatic cancer research and practice. The era of epigenetics has emerged strongly with well-justified and energetic beginnings, which will continue into a frontier area for pancreatic cancer research. The reversibility of the epigenetic changes, in itself, makes the journey worthwhile; however,

further insights into the mechanisms behind pancreatic cancer make the journey indisputable.

Box 1 Key Research Points

- The field of epigenetics has evolved from the fusion of studies on RNA polymerase II transcription and chromatin. The current theoretical framework in this field has been distilled from different paradigms, which have evolved during almost half a century with some replacement of each other.
- Pancreatic cells are excellent models for developing knowledge of three types of transcriptional events, namely basal transcription, activated transcription (e.g., growth factor-inducible), and tissue-specific gene expression (e.g., secretory granule enzymes).
- Studies on chromatin dynamics, including noncoding RNAs as well as nuclear structure and shape in pancreatic cells continue growing. The emerging data from these studies are benefiting not only this field, but extending the knowledge of the biology of other cells in the body. In addition, current evidence links these phenomena to development, homeostasis control and diseases. Therefore, this area may constitute one of the most promising in basic and translational pancreatic cell research.

Box 2 Future Scientific Directions

- Epigenetic mechanisms that are involved in stem cell biology, organ morphogenesis, and pancreatic cancer development constitute a new and very promising frontier. In particular, the discovery of how signaling and chromatin together determine cell fate during development and regeneration as well as how epigenetics contributes to the cancer phenotype is of paramount importance, biologically and pathobiologically.
- Cell-specific mechanisms for regulating gene expression are well advanced only in acinar cells. Therefore, more studies are necessary to understand the biology of ductal cells. In addition, epigenetic mechanisms are known to take part in the processes of pattern formation, such as branching morphogenesis, which is better understood in *Drosophila melanogaster* where chromatin-mediated effects play a significant role in this process. Therefore, studies on chromatin may aid in better understanding the formation of the pancreatic duct and its branching, which is of significant biomedical interest.
- Animal models for studying the genetic mechanisms necessary for the progression of pancreatic cancer have been a major contribution to the field of pancreatic cancer. Models for studying epigenetic effects in pancreatic cells must follow to understand the role of epigenetics in the pancreas at the whole organism level.

Box 3 Clinical Implications

- The revised "holo-genetic model for pancreatic cancer" covered in this chapter may help to guide future research in pancreatic cancer in a similarly productive manner to the guidance provided by the original genetic model for pancreatic cancer.
- It would be important to map key epigenetic changes that occur in the sequence of PanIN lesions along with the known mutations, to develop a better understanding of their potential mechanistic interrelationship. Therefore, development of new markers with good predictive value for whether an earlier PanIN has the potential to transform into another more malignant lesion would be beneficial.
- The most relevant characteristic of epigenetics, which is extremely attractive for therapeutic purposes, is its reversibility. Due to the difficulties surrounding gene replacement, it is likely that gene therapy for pancreatic cancer will remain, at least for a while, a hard-to-reach ideal. Therefore, due to its reversibility, epigenetics may provide attainable useful tools for chemoprevention and chemotherapy.
- In general, nuclear proteins and noncoding RNAs, which are shed by tumors into the bloodstream and are specific to detect pancreatic cancer, may be another prolific area of investigation with a great impact on diagnostics.

Cross-References

- ▶ Animal modeling of Pancreatitis-to-Cancer Progression
- ▶ Epigenetic Pharmacology
- ▶ Hedgehog Signaling Plays a Dual Role in Pancreatic Carcinogenesis
- ▶ Mouse Models of Pancreatic Exocrine Cancer
- ▶ Precision Medicine Based on Next-Generation Sequencing and Master Controllers
- ▶ Role of Tumor-Stromal Interactions in Pancreatic Cancer Invasion and Metastases
- ▶ The Molecular Pathology of Precursor Lesions of Pancreatic Cancer

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Molecular Pathology of Pancreatic Endocrine Tumors

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Abstract

The molecular biology of pancreatic neuroendocrine tumors (pNETs) carcinogenesis is poorly understood and is generally different from that of exocrine pancreatic neoplasms. pNETs represent a rare group of neoplasms with heterogeneous clinicopathological features. They are generally sporadic but can also arise within very rare hereditary syndromes, such as multiple endocrine neoplasia type I (MEN-I), von Hippel-Lindau disease (VHL), neurofibromatosis type 1 (NF1), and tuberous sclerosis complex (TSC). In these syndromes although a specific genotype/phenotype association with pNETs has been described, exact mechanisms leading to tumors development are still debated. Some clinical and biological features of pNETs associated with hereditary syndromes are similar in sporadic cases.

The presence of germline mutations has been indeed recently proved also in a high proportion of sporadic pNETs (17%) by whole genoming sequencing. These mutations include (beyond the well-known MEN1 and VHL) also other genes (such as BRCA2, or other of the mTOR pathway). Overall, main genomic changes involve gain of 17q, 7q, 20q, 9p, 7p, 9q and loss of 11q, 6q, 11p, 3p, 1p, 10q, 1q that identify the region of putative candidate oncogenes or tumor suppressor genes (TSGs) respectively. For some of them a possible relevant prognostic role has been described. "Classical" oncogenes involved in exocrine neoplasms (k-Ras, c-Jun, c-Fos) are of limited relevance in pNETs; on the contrary, overexpression of Src-like kinases and cyclin DI oncogene (CCNDI) has been described. As for TSGs, p53, DPC4/Smad, and Rb are not implicated in pNETs tumorigenesis, while for $p16^{INK4a}$, TIMP-3, RASSF1A, and hMLH1 more data are available, with data suggesting a role for methylation as silencing mechanism. Different molecular pathways and the role of tyrosine kinase receptors have also been investigated in pNETs (EGF, c-KIT) with interesting findings especially for VEGF and m-TOR, which encourage clinical development. Microarray analysis of expression profiles has recently been employed to investigate pNETs, with a number of different strategies, even if these studies suffer from a number of limitations, mainly related with the poor repeatability and the poor concordance between different studies. However, apart from methodological limits, molecular biology studies are needed to better know this group of neoplasms, aiming at identifying novel markers and targets for therapy also highlighting relations with clinical outcome. Besides biomarkers recent studies are currently focusing on the role of the immune system in tumor pathogenesis of pNETs, paving the way to a new therapeutic approach also in these rare tumors: the immunotherapy.

Keywords

Pancreatic neuroendocrine tumors · Carcinogenesis · Germline-mutations · Oncosuppressor genes · mTOR

Introduction

The molecular biology of pancreatic neuroendocrine tumors (pNETs) is poorly understood, and overall oncogenes and tumor suppressor genes (TSGs) more frequently involved in exocrine neoplasms, and particularly in pancreatic cancer, are not relevant. pNETs are generally sporadic, as their carcinogenesis is based on somatic mutations [1]. However, oncosuppressors responsible for pNETs can be involved by germline mutations, which are present also in a significant rate of sporadic pNETs [2]. This process may be spontaneous, without a previous family history, or more frequently inherited, as a part of well-described syndromes. The present paragraph will review in depth existing evidences for the molecular pathogenesis of pNETs, with a summary of data from studies of familial syndromes, genetic instability, as well as those examining the role of oncogenes, TSGs, and an insight into more recent microarray studies. A brief overview of the expression of growth factors and their receptors as possible therapeutic targets will also be presented.

Inherited Pancreatic Endocrine Tumors

The following hereditary syndromes have been associated with pNETs: multiple endocrine neoplasia type I (MEN-I), von Hippel-Lindau disease (VHL), von Recklinghausen's disease (neurofibromatosis 1 or NF1), and tuberous sclerosis complex (TSC) [3]. The latter three are phakomatoses, rare neurocutaneous syndromes characterized by uncontrolled growth of ectodermal tissues from which endocrine tumors arise.

The pNETs occurring in these hereditary forms are primarily nonfunctioning tumors or insulinomas, with different incidence, and do not differ from those detected as sporadic [3] (Table 1).

Multiple Endocrine Neoplasia Type I (MEN-I)

The most frequent inherited syndrome causing pNETs is MEN-I, a rare autosomal dominant disorder (incidence 1:20,000–40,000) clinically defined by the presence of two or more of the following neoplasms: gastroenteropancreatic neuroendocrine tumors, parathyroid gland adenomas, pituitary adenomas, with other neoplastic lesions (i.e., thyroid adenomas, multiple lipomas, bronchial or thymic carcinoids) occurring occasionally [4]. About 10% of pNETs occur as a part of MEN-I.

The MEN-I syndrome is the result of an inactivating mutation of the Menin gene, an oncosuppressor gene located on chromosome 11q13 [4].

This gene, consisting in 10 exons, encodes for a 68 KDa nuclear protein of 610 amino acids, named Menin. Menin functions include binding and inactivation

Table 1 Described syndromes associated with inherited pancreatic endocrine tumors, including clinical features and molecular defects

Syndrome	Gene	Gene function main molecular consequences	Major clinical features	Patients with pNETs (%)	pNETs subtype	Metastatic pNETs (%)
Multiple endocrine neoplasia type I	Menin (11q13)	Oncosuppressor deregulation of JunD, SMAD3 $p27^{KIPI}$ $p18^{Ink4c}$	2 or more between:	20–60%	80% NF	<10%
					15% insulinomas	
					3% glucagonomas	
			(a) GEP-NET		1% gastrinomas and vipomas	
			(b) Parathyroid adenomas			
			(c) Pituitary adenoma			
von Hippel-Lindau disease	VHL (3p25-26)	Oncosuppressor overexpression of HIF and VEGF	1 or 2 between:	5–17%	80–100% NF	<10%
			(a) Retinal or cerebellar hemangioblastomas			
			(b) Renal cell carcinoma			
			(c) Pheochromocytoma			
Von Recklinghausen's disease	NF1 (17q11.2)	Oncosuppressor deregulation of Ras pathway mTOR	(a) Café-au-lait skin spots	Rare	Insulinomas and somatostinomas	–
			(b) Neurofibromas of any type and localization			
Tuberous sclerosis complex	TSC 1 (9q34) TSC2 (16p13.3)	Oncosuppressor deregulation of mTOR pathway	(a) Skin alterations	Very rare	Mainly NF	–
			(b) Renal angiomyolipomas			
			(c) Multiple and diffuse hamartomas			
			(d) Neurological alteration			

of many nuclear transcription factors (especially JunD but also SMAD3, mSin3a, and trithorax family histone methyltransferase complex), upregulation of cell cycle inhibitors expression ($p27^{KIPI}$ and $p18^{Ink4c}$), and influence on DNA repair process, all of which result in inhibition of cellular proliferation [5–8].

The spectrum of possible mutations is greatly various. In the last decade, more than 1,300 germline variants (the half of which with pathological effect) have been identified, and 10–12% of them occur without a positive family history. Some 23% are nonsense mutations, 9% splicing-site mutations, 41% frameshift deletions or insertions, 6% in-frame deletions or insertions, 20% missense mutations, and 1% whole or partial gene deletions [4].

Even though any genotype/phenotype association with pNETs have been described, the exact mechanism leading to the neoplasia is still debated and the role of Menin on cell cycle negative control and DNA stability is somehow controversial.

Gene mapping in MEN-I patients have shown loss of heterozygosis (LOH) in half of the cases, confirming the oncosuppressor function of Menin and the tumorigenesis Knudson's two-hit hypothesis. LOH of the Menin gene and other somatic mutations on wild-type allele behave as a second hit after a first hit germline, inherited mutation. LOH on Menin allele, as described in sporadic pNETs, can also involve other terminal region of 11q, suggesting implications of additional genes in neoplastic development and progression. A heterogeneity among tumors even in the same patient, suggesting that different tumor-specific tumorigenic mechanisms may contribute to the pathogenesis of MEN1 tumors. The present study supports the clinical applicability of the WES strategy to research on multiple tumor samples and blood [9, 10].

pNETs patients with pathological Menin gene mutation do not differ from sporadic forms in terms of clinical features (age of onset, hormone and/or neoplasia-related symptoms), but only 10% develop metastases, especially in the case of tumors larger than 3–5 cm (irrespectively to its histotype) [1, 3].

In up to 80–90% of cases, endocrine pancreatic involvement consists in endocrine islet cell hyperplasia, without somatic LOH on Menin, and microadenomatosis (multiple indolent tumors <5 mm). These latter kind of lesions are characterized by trabecular structure and distinctive stroma, and, in spite of being asymptomatic and without metastases, in about 50% of the cases LOH of Menin gene is detectable [11–13].

In a variable percentage of MEN-I patients (20–60%), microadenomatosis is associated to one or multiple pancreatic "macro-tumors," which are larger than 5 mm but less than 3–5 cm. These neoplasms are NF pNETs in about 80% of cases, 15–20% insulinomas, 3% glucagonomas, and rarely VIPomas or gastrinomas [1–3].

These tumors are often clinically silent and just 10% of cases lead to metastases, but they are often associated with other symptomatic more aggressive gastrointestinal neuroendocrine tumors, especially duodenal gastrinomas and somatostatinomas [3, 14, 15].

In fact, although 20–60% of MEN-I patients have Zollinger-Ellison syndrome (20–40% associated with gastric carcinoid type II), gastrinomas arise far more

frequently in the duodenum as single or multiple small tumors (not unfrequently undetectable) rather than as pNETs [3, 16, 17].

Von Hippel-Lindau Disease (VHL)

pNETs also occur in a significant percentage of individuals affected by Von Hippel-Lindau disease (VHL). It is a very rare (1:30,000–1:50,000) autosomal dominant phakomatosis with a variable phenotype characterized by the presence of at least one of these major manifestations: single retinal or cerebellar hemangioblastoma (HB), renal cell carcinoma (RCC) or pheochromocytoma and other more rare multiorgan lesions such as pancreatic cysts or pNETs, renal cysts, endolymphatic sac tumors, epididymal papillary cystoadenomas, paragangliomas, polycythemia, and other rare tumors [18].

The gene responsible for this disease is VHL gene, an oncosuppressor of three exons located on 3p25-26 that by alternative splicing can encode for two proteins (pVHL), respectively of 213 and 160 amino acids [18].

The two VHL products accomplish to similar activities in the cytoplasm; in particular, they make an ubiquitin complex with cullin-2, Rbx1, and elongins B named VBC, which in case of normoxia binds and inactivates hypoxia-inducible factor (HIF) [14].

Inactivating mutation of VHL gene causes an overexpression of HIF, especially of vascular endothelial growth factor which lines to tumorigenesis [15].

Until now, more than 300 germline mutations have been found, 60% of which are truncating or missense mutations while 40% are deletions. These mutations are associated with different phenotypical expressions: only patients with missense mutations develop pheochromocytoma (VHL type 2) associated (2b) or not (2a) to RCC, whereas patients affected by other mutations will develop the remaining related disease manifestations (VHL type 1) [15, 18].

Disease penetrance grows by age (90% at 65 years), as germline mutations have to be followed by another somatic event in the wild-type allele.

As far as pNETs, LOH in the VHL allele or, less frequently, methylation or neomutation are frequent findings [15, 19]. Indeed, pancreatic involvement by multiple indolent cysts is typical of VHL (50–75%), but pNETs are also frequent (5–17%) [20].

Strict associations between specific mutations and phenotypic expression of pNETs have been reported, but tumor cells show a typical LOH in chromosome 3p which is not limited to the VHL gene, but also involves other adjacent genes (such as not papillary renal carcinoma-1) possibly implicated with tumorigenesis and progression [20].

Biological and clinical features of VHL-associated pNETs are similar to sporadic forms: they are typically nonfunctioning and asymptomatic, generally expressing somatostatin receptors and in 30–50% of cases are multifocal in the pancreas [3, 20, 21].

However, pNETs arising in VHL disease are usually small (<2–3 cm) and without liver metastases in about 80–90%, with a consequent better prognosis

compared to sporadic ones. This difference is most likely due to earlier detection (at a mean of 35 vs. 58 years) thanks to investigations due to other malignancies' symptoms [2, 3, 21].

Von Recklinghausen's Disease or Neurofibromatosis Type 1 (NF-1)

Occurrence of gastroenteropancreatic NETs in NF-1 is less frequent than in MEN-I and VHL disease, and in particular the rate of pNET is very low [22].

NF-1 is an autosomal dominant phakomatoses (1:3,000–1:4,000) with high penetrance, defined by multiple café-au-lait skin spots, neurofibromas of any type and localization (10% malignant), and characterized by predisposition to various other malignancies development (3–30%) such as gliomas, myeloid leukemia, and pheochromocytoma [23].

NF-1 arises from mutation of the NF-1 gene, a large oncosuppressor of 50 exons located on the 17q11.2 chromosome. Its product, called neurofibromin, is a GTPase acting as a negative regulator of mitonegic Ras pathway, especially of the mTOR signaling [24].

Many NF-1 gene mutations have been identified, of which up to 50% arising "de novo"; however, all the significant genotype/phenotype association have been demonstrated [23].

Rate of associated pNETs is undeterminable [3, 25–27]. They arise from germline NF1 mutation and deletion; insulinomas and somatostatinomas are similar to sporadic forms as in the tumor cells there is low expression of NF-1. The risk of pNETs development is often increased in this disease, probably because of mTOR pathway upregulation; however, more cases are needed to study the genotype/phenotype relation.

Tuberous Sclerosis Complex (TSC)

The rarest inherited disease associated with gastroenteropancreatic NETs is TSC. This phakomatosis (1:10,000) is a hereditary multiorgan disease transmitted by autosomal dominant inheritance. TSC has a 100% penetrance and a highly variable expression; clinical manifestations are typical skin alterations, renal angiomyolipomas, multiple and diffuse hamartomas, mental retardation, and neurological alterations. pNETs are occasionally associated [28].

Two genes are responsible for this disease: TSC1 (9q34) and TSC2 (16p13.3) that respectively encode for hamartin and tuberin. These two proteins make a dimer that multi-modulates cell growth, interacting with phosphoinositide 3-kinase pathway-mTOR activity and insulin receptor signaling.

Several genotype/phenotype associations have been described and related to many different mutations (50% occurring de novo); somatic tumor cells show a secondary mutation or a large deletion, up to a complete LOH on the two alleles often involving large chromosomal region.

The described cases of pNETs associated with this disease are mainly nonfunctional, and few cases of insulinoma and somatostinoma, with a behavior similar to sporadic forms [5]. In particular, one case of pNETs described in literature, a nonfunctional tumor identified in a child, exhibited a TSC2 gene LOH; this confirms its oncosuppressor role, such as in other TSC-related neoplasm [29, 30].

Genetic Instability in Sporadic Pancreatic Endocrine Tumors

Genetic instability represents the necessary condition for tumor development, through the clonal expansion of cancerous cells that have acquired a selective advantage. Among the different events (point mutations, chromosomal rearrangements, gene amplifications, microsatellite sequences alterations, and epigenetic changes) occurring during the multistep process of somatic cells transformation, alterations in DNA copy number are the commonest events.

Allelic imbalances, that result from incorrect mitotic division and consequent abnormal chromosomal separation, may be revealed by a variety of methods including karyotyping, comparative genomic hybridization (CGH), microsatellite analysis, or, more recently, single nucleotide polymorphisms (SNPs) allelotyping.

Conventional CGH is a molecular cytogenetic genome-wide technique for the analysis of copy number changes in DNA of tumor cells. Through this method, differentially labeled test DNA and normal reference DNA are hybridized simultaneously to normal chromosome spreads and the hybridization is detected with two different fluorochromes. Regions of gain or loss of DNA sequences, such as deletions, duplications, or amplifications, are seen as changes in the ratio of the intensities of the two fluorochromes along the target chromosomes. In brief, the regions frequently identified with decreased copy number are likely to harbor tumor suppressor genes (TSGs), whereas regions with increased copy number may contain dominant oncogenes.

Furthermore, allelotyping, that is the systematic analysis of the allelic losses in single chromosomes thus exploring loss of heterozygosity (LOH), is another strategy to determine the most probable locus of a TSG: it can be based on polymorphic microsatellite DNA or on SNPs, assaying the frequency and extent of lost regions on all chromosomal arms. SNPs allelotyping is more sensitive than microsatellite analysis and is also useful to detect DNA copy number.

Genome-Wide Studies in Sporadic pNETs

During the last decade, several studies with different approaches have addressed to look for specific genomic defects in sporadic pNETs [31–42]. As shown in Table 2, CGH has been largely used to explore genetic aberrations. Most of the available data refer to small, heterogeneous tumor series and essentially regard well-differentiated pNETs. In addition, several different tumor classifications have been used by investigators in their studies during time making difficult a possible analysis of

Table 2 Main genome-wide studies of pNETs series

Method of study	N° pNETs	pNETs subtypes	Reference
CGH	12	10 NF,2 F	[31]
CGH	44	9 NF, 35 F	[32]
CGH	25	25 F	[33]
CGH	8	8 F	[34]
CGH	38	10 F, 28 F	[35]
CGH	45	14 NF, 31 F	[36]
CGH	9	3 NF,6 F	[37]
CGH	20	20 NF	[38]
CGH	62	62 F	[39]
Genome-wide allelotyping	28	7 NF, 21 F	[40]
Genome-wide allelotyping	32	32 NF	[41]
SNPs allelotyping	15	13 NF, 2 F	[42]
CGH	67	-	[43]
			[2]

CGH comparative genomic hybridization, *SNPs* single nucleotide polymorphisms, *NF* non-functioning, *F* functioning

pNETs subtypes. In this paragraph, data are presented separating nonfunctioning (NF-) from functioning pNETs (F-pNETs), and among these, further taking account of benign insulinomas, malignant insulinoma, and gastrinomas to possibly identify specific genomic patterns.

In the ten published studies [31, 32, 35–38, 40–43] of CGH/genomic wide-allelotyping, 101 NF pNETs have been studied (Tables 3 and 4). The most frequent findings were losses of 11q (38.6%), 6q (37.6%), 11p (33.7%), 3p (26.7%), 1p (27.7%), and 10q (25.7%), while the most frequent gains involved 17q (41%), 7q (35.9%), 12q (34.6%), 14q (34.6%), 4p (32%), and 20q (30.7%).

As for the 31 gastrinomas investigated in seven studies, loss of 3p (19%) and gain of 9p (29%) represented the most common chromosomal aberrations [31, 32, 34–37, 40].

In benign insulinomas (116 overall tumor samples in seven studies), most frequent losses were found on 11q (19%), Xq (18%), and 1p (17%), while most frequent gains regarded 9q (41%), 7p (20%), and 7q e 5q (both 19%). Malignant insulinomas (30 tumor samples), defined by the presence of loco-regional advanced or metastatic disease, harbored more genomic alterations than benign counterpart [32, 33, 35–37, 39, 40]. In particular, most frequent losses were found on 6q (70%), Y (43%), 2q (33%), 3q (30%), 6p (30%), 10q, 11p, 11q, and Xq (all 23%), while main gains involved 17q (57%), 17p (53%), 12q (53%), 14q (50%), 7q (47%), 20q, and 9q (43%).

The identification of gains and losses on chromosomal regions helps to highlight loci potentially containing putative oncogenes and TSGs. Tables 5 and 6 summarize main losses and gains, together with candidate TSGs and oncogenes, the associated disorders for which a pathogenetic link has been already described and, finally, the prognostic significance of the particular genetic change.

Table 3 Frequence of chromosomal losses (%) in pancreatic neuroendocrine tumor subtypes

		1p	1q	2p	2q	3p	3q	4p	4q	6p	6q	8p	8q	10p	10q	11p	11q	13q	15q	16p	16q	21p	21q	22p	22q	Xp	Xq	Y
	n°	%																										
NF	101	26	24	21	23	27	25	2.9	9.9	24	37.6	19	20	24.8	25.7	34	38.6	12	19	13.9	16.8	12	19.8	11	20	5	5	5
B Ins	116	17	15	0	5	0	3	8	9	0	3	4	7	0	0	15	19	5	0	1	1	0	0	0	4	13	18	7
M Ins	30	27	20	10	33	20	30	13	10	30	70	3.3	10	6.6	23	23	23	17	3.3	6.6	10	0	13.3	0	10	13	23	43
Gas	31	3	10	0	0	19	10	0	0	0	0	0	3	0	3	3	13	3	3	3	3	0	0	0	0	0	0	0

NF nonfunctioning, *B* or *M Ins* benign or malignant insulinoma, *Gas* gastrinoma

Table 4 Frequence of chromosomal gains (%) in pancreatic neuroendocrine tumor subtypes

		2q	3q	4p	4q	5p	5q	6q	7p	7q	8p	8q	9p	9q	11q	12p	12q	13p	13q	14p	14q	15q	16p	16q	17p	17q	18p	18q	19p	19q	20p	20q	Xp	Xq	Y
	n°	%																																	
NF	101	0	32	26	28	28	3	28	36	6	10	19	27	3	24	34	12	22	17	34	3	0	0	29	41	22	28	15	17	26	31	1.2	5	0	0
B Ins	107	0	1	2	1	13	19	5	20	19	1	1	6	41	3	3	4	0	1	3	8	11	0	0	3	5	3	2	10	10	5	12	5	1	2
M Ins	30	0	3	10	20	23	37	0	37	47	0	0	13	43	6	17	53	0	13	3	50	23	17	6	53	57	3	3	8	8	20	43	27	20	10
Gas	17	12	0	0	0	6	6	0	6	12	12	6	29	12	6	12	12	0	0	12	12	12	6	0	12	12	0	0	0	0	0	6	0	0	0

NF nonfunctioning, *B* or *M Ins* benign or malignant insulinoma, *Gas* gastrinoma

Table 5 Main losses in sporadic neuroendocrine tumor of the pancreas

Location	% Loss				Putative TSGs	Associated disorder	Prognostic relevance
	NF	B Ins	M Ins	Gas			
11q	38.6	19	23	13	MEN-1	MEN-1 syndrome	Presence in up to 37% of cases [2]
					PLCB3		
					SDHD	Intestinal carcinoids, paraganglioma, pheochromocytoma	
					TSG11	Nonsmall cell lung cancer	
					HHPT	Hereditary hyperparathyroid-jaw tumor syndrome	
					BRCC2	Breast cancer	
					ZW10		
6q	37.6	3	70	0	AIM 1	Melanoma	Associated with liver metastasis [32]
					CCNC		
					PTPRK		
					LOT-1	Transient neonatal diabetes mellitus	
					CX43	Oculodentodigital dysplasia, hypoplastic left heart syndrome, atrioventricular septal defect	
11p	33.7	15	23	3	WT1	Wilms tumor type 1, Denys-Drash syndrome, WAGR syndrome, Frasier syndrome, isolated diffuse mesangial sclerosis	
3p	26.7	0	20	19.4	VHL	Von Hippel Lindau syndrome, renal cell carcinoma	Associated with liver metastasis [32, 44]
					hMLH1	Colorectal cancer, HNPCC	
					RAR-β		
					B-Catenin	Digestive endocrine tumors	
					RASSF1A	Lung cancer	

(*continued*)

Table 5 (continued)

Location	% Loss						
	NF	B Ins	M Ins	Gas	Putative TSGs	Associated disorder	Prognostic relevance
1p	27.7	17	26.6	3	p73	None	Associated with liver metastasis [45]
					p18/INK4		
					RUNX3		
10q	25.7	0	23	3	MGMT	Endometrial k, follicular thyroid k, meningioma	
					PTEN		
1q	24	15	20	10	HHPT2	Hereditary hyperparathyroid-jaw tumor syndrome	Associated with metastases and aggressive growth [46–49]
						Several cancer cell lines	
					MDA7/ IL-24		

TSGs tumor suppressor genes, *NF* nonfunctioning, *B or M Ins* benign or malignant insulinoma, *Gas* gastrinoma

On the whole, NF-pNETs seem to present more genomic aberrations, then malignant insulinomas, with benign insulinomas and gastrinomas presenting the lowest amount of changes. This tendency is consistent with the finding by Speel and colleagues that pNETs larger than 2 cm exhibited significantly more aberrations than lesions smaller than 2 cm given that NF-pNETs are often larger than 2 cm at diagnosis [32].

All these observations strongly suggest that pNETs subtypes may evolve along different molecular pathways: deciphering their specific signatures would help to implement pNETs classification system, with obvious implications for a better understanding of this complex nosological entity.

Prognostic Relevance

Accumulated evidences showing that pNETs from patients with advanced disease harbored significantly higher numbers of genetic aberrations than tumors from patients with localized disease suggest that malignant progression of pNETs

Table 6 Main gains in sporadic neuroendocrine tumor of the pancreas

Location	% Gain				Putative oncogenes	Associated disorder	Prognostic relevance
	NF	B Ins	M Ins	Gas			
17q	41	5	57	12	Neu/ ERB2	Breast cancer	Associated with malignant behavior in tumors <2 cm [35]
7q	35.9	19	47	12	HGF C-MET	Gastric cancer, hepatocellular carcinoma	
Xq					ATRX/ DAXX	Alpha-thalassemia	Associated to reduced survival [43]
20q	30.7	12	43	6	STK15/ BTAK	Breast cancer, ovarian and digestive carcinomas	
9p	19.2	6	13	29	JAK2	Acute myelogenous leukemia, myeloproliferative disorder	
					Oncogene ovc	Ovarian carcinoma	
					RAGA		
7p	28	20	37	6	EGFR/ ERBB1	Bladder, breast, epidermoid carcinoma, glioblastoma	
9q	26.9	41	43	12	VAV2	Breast cancer, head and neck squamous carcinoma	
					CDK9		
					cABL	Chronic myeloid leukemia, insulinoma rat cell lines	
					NOTCH-1	SCLC, T cell acute lymphoblastic leukemia	
					LMX1B		

NF nonfunctioning, *B or M Ins* benign or malignant insulinoma, *Gas* gastrinoma

progression is driven by the progressive accumulation of multiple genetic changes [32, 36, 39, 50], as is also known to occur in other types of human carcinomas [46].

Another interesting issue is the possible relationship between molecular genetic defects (number and type of genomic changes) and tumor progression or malignancy in pNETs.

Several LOH studies [45, 47–49], using microsatellite markers, demonstrated that LOH at *chromosome 1*, and in particular of its long arm, is a common event among pNETs subtypes (12/27 gastrinomas, 35/40 insulinomas, 10/29 different pNETs subtypes) and was significantly associated with the presence of hepatic metastases regardless of tumor type. Moreover, Chen and colleagues (2003) found in their series of gastrinomas that allelic loss at 1q31-32 as well as 1q21-23 significantly correlated with tumor aggressive growth and postoperative development of liver metastases [48]. Likewise, Yang and colleagues (2005) reported high frequency of LOH at 1q 21.3-23.2 and 1q31.3, significantly associated with malignancy of insulinomas suggesting in these two regions the presence of putative tumor suppressor genes important for aggressive growth of these tumors [49]. Although these two studies narrowed region of potential candidate genes, to date actual genes involved remain undefined (Table 5).

As for *chromosome 3*, LOH was demonstrated to be a common event (frequency ranged from 33% to 83%) in pNETs regardless of tumor subtypes and its frequency was significantly higher in malignant than in benign neoplasms, on the whole finding a correlation with clinically metastatic disease in several studies [44, 51–53]. As common deleted regions were different (3p14.2-21; 3p25.3-p23; 3q27-qter, all outside of the VHL locus) in the same studies, different putative tumor suppressor genes other than VHL on chromosome 3 may play a role in the latest steps of tumorigenesis of sporadic pNETs.

Only one LOH study reported by Barghorn and colleagues (2001) described allelic loss at *chromosome 6* in 62.2% of cases in a heterogeneous cohort of pNETs, the majority of which were insulinomas and NF-pNETs (with common deleted regions mapped at 6q22.1 and 6q23-q24), and it was significantly more common in tumors larger than 2 cm in diameter than below this threshold as well as in malignant than in benign tumors [43]. Previously, Speel and colleagues (1999) had reported an overall loss at 6q in 39% of pNETs (with a common deleted region at 6q21-22) and in all of six insulinomas, again indicating a locus harboring a potential TSG involved in tumor development [32]. To further support this hypothesis, combined data from abovementioned genome-wide studies show that 6q loss occurs in 70% of malignant insulinomas and in 37.6% of NF-pNETs, as shown in Table 2 .

Chromosome 17. In a study of 20 mixed functioning and nonfunctioning pancreatic endocrine tumors, Beghelli and colleagues (1998) found allelic losses on 17p13 in ~24% of the chromosomal loci analyzed with a higher frequency of allelic losses significantly associated with a high proliferation index and malignancy of the tumors [54]. Moreover, the absence of p53 gene mutations in nearly all these tumors

suggests the existence of another tumor suppressor gene in the same chromosomal area. However, according to genomic-wide studies, loss of 17p is a rare event (<10%) and probably does not play a central role in the majority of endocrine tumors development. On the opposite, gain of 17q is a frequent event, especially in malignant insulinomas (>50%). The oncogene Her-2/Neu, frequently overexpressed in breast and esophageal cancer which is identified as more aggressive phenotype, is located on chromosome 17q21. Her-2/Neu gene amplifications were identified in 40% of 11 gastrinomas [55], the majority of which were locally advanced or metastatic, while in another study by [56] the same gene was amplificated in 14% of 43 gastrinomas and this time higher mRNA levels in tumor cells were correlated with liver metastases [56].

LOH on *chromosome 22q* was detected in 14 of 15 insulinomas (93%) by Wild and colleagues (2001). The shortest region of overlap implicated a deletion at 22q12.1-q12.2 where hSNF5/INI1 gene is located but no alteration was identified by single strand conformational polymorphism analysis, direct DNA sequencing, or RNA expression analysis [57]. The same group [58] described LOH on chromosome 22q in 22 of 23 pNETs (including nonfunctioning tumors, gastrinomas, and vipomas) showing a LOH rate of 85% at locus 22q12.1, with LOH strongly correlated with the presence or the development of distant metastases [58]. Moreover, LOH on 22q12.3 was significantly associated with distant metastases, an area where two putative candidate gene are located, that is, synapsin3 (SYN3) and tissue inhibitor of metalloproteinase-3 (TIMP-3). Also in this instance, genome-wide studies tend to underestimate genetic changes: in particular, loss of 22q was found in ~20% of NF-pNETs and in less than 10% of other pNETs subtypes.

Sex Chromosomes. According to combined data from genome-wide studies reported, Xq loss mainly occurs in insulinomas (~20% of cases) and one CGH-study also noted an association between Xq loss metastatic disease, raising the hypothesis that X chromosome changes plays a role in defining the more aggressive nature of endocrine lesions [32, 43].

Aberration of X chromosome has been described mainly in gastric carcinoids and pNETs, and in malignant compared with benign endocrine tumors. Pizzi et al. [59] comparing pNETs and endocrine tumors of the ileum and appendix noted that LOH on chromosome X was evident in 60% of malignant gastric and pancreatic tumors but in only 4.5% of benign tumors. Similarly, none of the benign midgut tumors exhibited X chromosome LOH, whereas 15% of malignant tumors contained this aberration [59]. On the whole, an association between X chromosome LOH and malignancy clearly has been found. In LOH analysis, allelic losses on X chromosome were revealed in 50% of type III gastric carcinoids, but not in type I tumors. Again, tumors that exhibited LOH were associated with metastasis [60]. Also in a series of 16 female patients with gastrinomas reported by Chen et al. 56% presented X chromosome LOH, was significantly associated with aggressive postoperative tumor growth and with increased primary tumor size [61]. Missiaglia and colleagues (2002), in their microsatellite and FISH analysis extended to chromosome Y, described that pNETs from females had loss of chromosome X in 40% of cases whereas pNETs from males showed loss of chromosome Y in 36% of case but never had loss of the X chromosome [62]. A significant association of sex chromosome

loss with metastases, local invasion Ki-67 > 5% was also described. Sex chromosome loss was found to be an independent variable associated with a shorter survival period and an increased risk of death of approximately fourfold.

Recently, in a comparative LOH analysis on X chromosome by Azzoni et al. [63] higher rate of allelic loss was found in poorly differentiated endocrine carcinomas than in well-differentiated endocrine carcinomas with two chromosomal regions, Xq25 and Xq26 showing LOH with a relatively high frequency [63]. Candidate tumor suppressor genes mapping at Xq25 are ODZ1, encoding Tenascin, a glycoprotein of the extracellular matrix involved in morphogenetic movements, tissue repair and tumor spreading and SH2D1A, whose mutation was described in X-linked lymphoproliferative disease and in non-Hodgkin Lymphomas [64]; while potential tumor suppressor genes for Xq26 are MEF, a transcription factor capable to suppress the transcription of the genes encoding for the matrix metalloproteinases, MMP-2 and MMP-9, and interleukin-8 as demonstrated in cell lines of human nonsmall cell lung carcinoma [65]; and GPC-3, a heparan sulfate proteoglycan linked to the cell membrane, involved in the progression of several types of malignant tumors, including mesotheliomas, ovarian, and lung carcinomas [66].

Loss of DAXX or ATRX protein and alternative lengthening of telomeres have also been proved to show a prognostic meaning in pNET cases. They were indeed associated to tumor stage, relapse-free survival, and decreased time of tumor-associated survival in 243 patients affected by pNETs [43].

Final Considerations

The limited resolution of the conventional CGH method, its low reliability (emerged from the observation that some regions – 1p32- pter, 16p, 19, and 22 – showed gains in negative control experiments), and its feature to be a laborious method remain the principal limits. On the other hand, LOH analysis, depending on number and type of microsatellite markers used, often offers contradictory results. For this reason, caution is needed in interpreting their results, awaiting further studies to confirm available data.

Array-CGH technology can improve the resolution of conventional CGH on metaphase chromosomes from 5 to 10 Mb to $\leq$1 Mb on arrayed DNA. In a series of 27 insulinomas, Jonkers and colleagues (2006) performed a genome-wide array-based CGH analysis detecting in >50% of cases loss of chromosomes 11q and 22q and gains of chromosome 9q with the first two alterations only partially identified before by conventional CGH (11q loss and 22q loss were found in ~20% and ~10% of benign and malignant insulinomas, respectively) [67].

The chromosomal regions of interest included 11q24.1 (56%), 22q13.1 (67%), 22q13.31 (56%), and 9q32 (63%). Comparing their alteration frequencies in tumors with benign, uncertain, and malignant behavior according the most recent WHO classification, the authors suggest that gain of 9q32 and loss of 22q13.1 are early genetic events in insulinomas, occurring independently of the other alterations. Finally, in this study further evidence was found for the accumulation of chromosomal alterations which run parallel with increasing malignant potential.

Genetic Alterations of Oncogenes and Tumor Suppressor Genes, and Expression of Growth Factors and Their Receptors

Oncogenes

The role of *k-Ras* has been investigated by a number of authors, with findings suggesting limited relevance if any, thus differentiating pancreatic endocrine neoplasms from the exocrine counterpart. *K-ras* mutations were found in a risible proportion of cases [50, 54, 55, 68–72], without any significant clinical association. Not surprisingly, the BRAF gene, one of the human isoforms of RAF, which is activated by ras, does not seem to have a role in tumorigenesis of pNETs [73]. However, a possible role for the ras signaling pathway in pNETs may depend on inactivation of the TSG RASSF1 (see below).

Similarly, there is limited evidence for a role of either *c-Jun* or *c-Fos* [71, 74]. On the other hand, *c-Myc* is overexpressed in most studies either at the RNA or protein level [50, 68, 75, 76]. The proto-oncogene *Bcl-2*, which acts as an antiapoptotic factor, has been detected in up to 45% of examined pNETs samples [75]; however, there are no data examining the overall balance of the pro/antiapoptotic machinery in pNETs.

Src is a family of proto-oncogenic nonreceptor tyrosine kinase including nine members. *Src-like kinases* act downstream of growth factor receptors and integrins transmitting messages that are crucial for several aspects of cell growth and metabolism, as for example cell cycle regulation, cell adhesion, and motility. Overexpression of *Lck*, a member of *Src* family, has been recently demonstrated in metastatic progressive pNETs in a microarray study [74]. The expression and activity of *Src* have been also described in pNETs cell lines and tissues, and inhibition of *Src* activity has been shown to interfere with adhesion, spreading, and migration of cells [77].

As far as cell cycle, although animals with constitutive activation of CDK4 develop pNETs [78], mutations have not been found in insulinomas [79]. A more relevant role for the cyclin DI oncogene (CCNDI) is suggested by findings of its overexpression and relation with disease stage [80, 81].

The Wnt signaling pathway is relevant for a number of neoplasms, and β-catenin activation is frequently detected in such cancers. However, no mutations of the β-catenin gene have been detected in a study including 108 pNETs, and nuclear accumulation of the β-catenin protein seems a rare and late event [82].

In a further study, 52% of pNETs showed abnormal β-catenin staining, which was related with loss of normal E-cadherin staining and more aggressive behavior [83].

Tumor Suppressor Genes

The role of MEN-I and VHL mutations, either in genetic or sporadic forms, has been summarized in the previous paragraphs.

The role of the *p53* TSG has been investigated in a wide number of studies. A rationale for such investigations comes from studies of mice with *p53* mutations and

pNETs development. However, most studies found no mutations of *p53* and/or no overexpression of the mutated protein in human pNETs [50, 54, 55, 68, 69, 72, 73, 84–87]. These data suggest that findings of LOH at 17q13 may be related with other unknown TSGs.

Similarly, although LOH at 18q is fairly frequent in pNETs, the DPC4/Smad gene has not been found to be mutated in the majority of published papers [50, 68, 88], and the retinoblastoma TSG (Rb) is also not implicated [89].

On the other hand, the p1*6 INK4a* TSG, which encodes for an inhibitor of CDK4, seems relevant for at least a portion of pNETs. Particularly, inactivation of *p16*, either by mutations or by methylation is common in gastrinomas, but less frequent in NF-pNETs and insulinomas [55, 68, 90, 91].

The expression of the putative tumor suppressor gene tissue inhibitor of metalloproteinase-3 (TIMP-3) has been found to be altered by either promoter hypermethylation or homozygous deletion. The predominant TIMP-3 was described in 44% of examined pNETs, with as significant relation with the metastatic process [92].

The Ras-association domain family 1A (RASSF1A) is a TSG, interacting with ras. It is inactivated in a variety of solid tumors, usually by epigenetic silencing of the promoter or by loss at 3p21.3. RASSF1A induces cell cycle arrest through inhibition of cyclin D1 accumulation. RASSF1A hypermethylation was detected in 10 out of 12 (83%) endocrine tumors [93], and in a further publication RASSF1A silencing by methylation and 3p21.3 deletion was associated with tumors from foregut only, and with malignant behavior [94].

Loss of expression of the p27 protein has instead been paradoxically related with well-differentiated pNETs, with most indolent features, while its expression was associated with metastatic disease [95].

The aberrant promoter methylation of the mismatch repair gene, hMLH1, is associated with microsatellite instability (MSI). Hypermethylation of the hMLH1 promoter has been found in 23% of pNETs. Some 50% of hMLH1-methylated pNETs were found to be microsatellite unstable, and MSI was restricted to pNETs with hMLH1 hypermethylation. Tumors with MSI-positive had a better survival compared with MSI-negative [96].

Growth Factors and Their Receptors (Receptor Tyrosine Kinases)

The expression of growth factors, and their receptors, generally tyrosine kinases, is an interesting issue and offers the opportunity for targeted therapy. Angiogenesis has been studied in depth in transgenic mouse model (Rip1-Tag2) in which mice develop pNETs [97]. Although pNETs are highly vascular, some studies have suggested that they express VEGF, which correlates with a more aggressive tumor [98], while others detailed how pNETs present a wide range of microvascular density (MVD) according to the malignant potential, with malignant tumors showing lower MVD and VEGF expression than benign ones [99].

The surface of pNETs cells presents several other growth factor receptors, including receptor tyrosine kinases such as the epidermal growth factor receptor

(EGFR), the stem cell factor (SCF) receptor c-KIT, and the platelet derived growth factor receptors (PDGFR) [100–103].

The EGFR (ErbB-1) is a member of a receptor tyrosine kinase family also including HER2/Neu (ErbB-2), HER-3 (ErbB-3), and HER-4 (ErbB-4), whose activation after interaction with their ligands leads to a number of downstream cascade molecular events involving cell proliferation and transformation. Although the expression of the EGFR and its phosphorylation seems more relevant in carcinoids than pNETs, phosphorylated-EGFR expression was found to be an unfavorable prognostic marker only in pNETs [104]. As far as other members of the Erb family, the expression and amplification of HER-2/Neu were explored in patients with gastrinoma, with relevant data presented above [55, 56].

c-KIT (CD117) is a type III tyrosine kinase receptor which, once activated by its ligand, stem cell factor (SCF), induces dimerization and autophosphorylation of the receptor at specific tyrosine regions, which acts as docking sites for other intracitosolic proteins important for intracellular signal transduction. Abnormal expression of c-KIT and/or SCF has been described in a variety of solid tumors, and activating mutations of c-KIT are a typical feature of gastrointestinal stromal tumors (GIST). Several studies have investigated the expression of c-KIT, together with other receptor tyrosine kinases in gastroenteropancreatic endocrine tumors, by immunohistochemistry [102, 105]. The results are inconsistent and, as hypothesized for other cancer types, inter-studies disagreement may be explained by different antibodies employed or different immunohistochemistry protocols.

A recent study including 98 pNET samples [2] has proved that sporadic pNETs contain germline mutations in about 17% of patients. These mostly interest genes involved in four main pathways: chromatin remodeling, DNA damage repair, activation of mTOR signaling (including previously undescribed EWSR1 gene fusions), and telomere maintenance, hypoxia, and HIF signaling. Also further mutations involving MUTYH, APOBEC, und BRCA have been described, paving the way to further molecular targets for therapeutic approach.

The (PI_3K)/Protein Kinase B/AKT/mTOR Pathway

The mammalian target of rapamycin (mTOR) is a serine-threonine kinase involved in the mechanisms of regulation of cell growth and death through apoptosis. It plays a critical role in transducing a number of different proliferative signals mediated through the phosphatidylinositol 3 kinase (PI_3K)/protein kinase B (AKT) pathway, principally by activating downstream protein kinases that are required for both ribosomal biosynthesis and translation of key mRNAs of proteins required for cell cycle progression.

The signaling pathways upstream of mTOR include several tumor suppressors, such as PTEN, NF1, the kinase LKB1, and oncogenes such as Ras and Raf. mTOR also mediates signaling downstream of a number of growth factors such as IGF-1 and VEGF (Fig. 1). These signaling pathways converge on the tuberous sclerosis

complex (TSC1/TSC2), which inhibits the mTOR activator Rheb, a small GTPase. In turn, activation of the mTOR pathway enhances the activity of HIF1α and of VEGF itself [106, 107].

Tumors exhibiting constitutively activated PI_3K/AKT/mTOR signaling due to mutations or loss of the abovementioned tumor suppressor genes (PTEN or TSC), or overexpression of upstream genes, are potentially susceptible to mTOR inhibitors, therefore making the investigation of this pathway particularly interesting for pNETs.

Microarray Studies

Global expression profiling has been often employed in the past decade to better understand molecular changes occurring in a number of tumors. This approach has been proved useful to identify novel markers and targets for therapy or to highlight relations with clinical outcome.

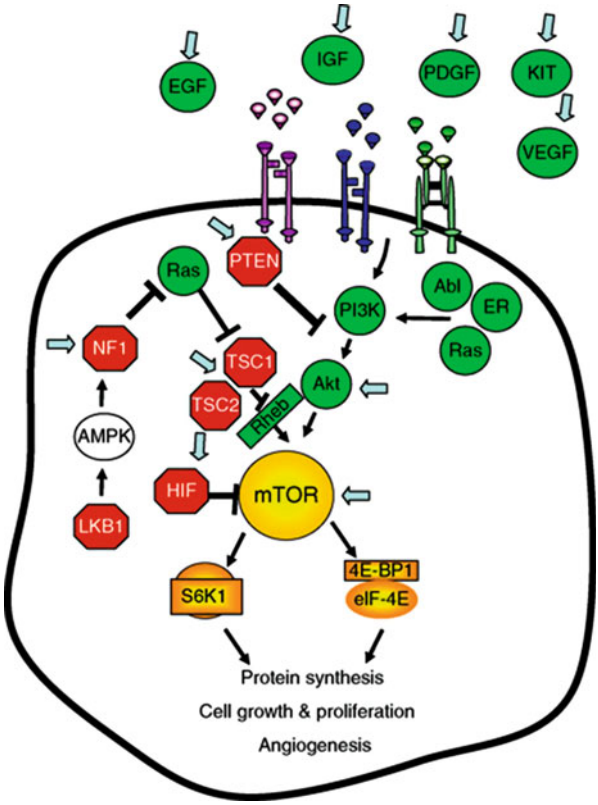

Fig. 1 Schematic representation of the PI_3K/AKT/mTOR pathway. Green color indicates overexpression or activation, and red color indicates reduced expression or deactivating mutations. Overall, the balance of such events suggests an important role for this signaling pathway in pNETs. Notably, mutations of TSC1/TSC2 and PTEN may reduce the negative effect of hypoxia on the mTOR pathway

However, microarray studies suffer a number of limitations, mainly related with the poor repeatability, and the poor concordance between different studies [108].

Microarray analysis of expression profiles has recently been employed to investigate pNETs, with a number of different strategies. These studies are summarized in Table 7 [74, 109–114].

Overall, the studies differ significantly in terms of different samples and design, different platforms and statistical/bioinformatics methods. Two of the studies [74, 113] employed a wider platform. Two main different design subgroups can be identified: (1) comparison of pNETs samples versus purified pancreatic islets [74, 109, 110] and (2) comparison of metastatic versus non-metastatic pNETs [112–114]. One other study compared expression profiles of pooled biopsy material of pNETs with that obtained from other pancreatic pathologies and normal pancreata [111], making its comparison with the other studies of poor sense. However, some of these studies did not provide clinical or histopathologic data sufficient to determine the clinical behavior of the investigated patients, and only one of the studies also compared primary lesions versus liver metastases [74], with findings suggesting a striking similarity between matched primaries and metastases.

Overall, none of the studies could identify novel dysregulated genes associated with a certain clinical behavior or with prognosis or response to treatment. The overlap between the different gene lists is very poor, as previously reported for pancreatic adenocarcinoma [115]. However, some interesting candidates for further evaluation as prognostic factors or therapeutic factors may have been identified.

A single paper examined the expression of microRNAs in pNETs [116]. MicroRNAs are small noncoding RNAs able to regulate gene expression by targeting specific mRNAs for degradation or translation inhibition. A role for microRNAs in tumor development and progression has been ascertained for many human cancers including pancreatic adenocarcinoma. Using a specific custom microarray, Roldo et al. explored the global microRNA expression of 40 pNETs (12 insulinomas, 28 non functioning tumors) compared to normal pancreas, and showed that a common pattern of microRNA distinguishes pNETs from normal pancreas. Specific microRNAs were identified, such as miR-204, primarily expressed in insulinomas and miR-21 which was strongly associated with both high Ki67 and liver metastases.

Conclusion

Research has made significant progresses in the knowledge of pNETs' molecular biology but still the carcinogenesis involves mechanisms that need to be clarified. This multistep process may involve mutations of oncosuppressors genes, as well as germline mutations, which have been identified also in sporadic tumors. Further studies have to focus on immunotherapy and on the development of new target therapies to offer new treatment options to these patients.

Table 7 Summary of gene expression profile studies of pancreatic endocrine tumors

Author [reference]	Samples	Comparison(s)	Platform	Upregulated genes	Downregulated genes	Relevant genes	Confirmation
Capurso [74]	13 NF pNETs (8 primary, 5 metastasis), 3 cell lines (BON CM QGP), 4 purified islets	1. pNETs versus islets	Affymetrix U133A + B	668	323	LCK, BIN1, BST2, SERPINA10	IHC qRT-PCR
		2. Primary versus Metastases		_	_		
Maitra [109]	8 NF pNETs, 3 purified islets	pNETs versus islets	Affymetrix U133A	66	119	IGFBP3, fibronectin, MIC2, p21	IHC
Dilley [110]	8 pNETs samples from 6 MEN-I patients (2 insulinomas, 2 NF, 1 vipoma, 1 gastrinoma), 4 purified islets	pNETs versus islets	Affymetrix U95AV2	45	148	IER3, IAPP, SST, PHLDA2	qRT-PCR
Bloomston [111]	Pooled biopsies from 9 pNETs, normal pancreas, PDAC, CP	pNETs versus normal pancreas	Affymetrix U133A	Ns	Ns	ANG2, NPDC1, ELOVL4, CALCR	IHC RT-PCR
Duerr [112]	24 pNETs (9 insulinomas, 4 NF, 3 gastrinoma, 1 glucagonoma, 1 ACTHoma, 1 PTHRPoma), 6 GI carcinoids	1. 12 WDETs versus 7 WDECs	Affymetrix U133A	71	41	FEV, NR4A2, ADCY2, GADD45β	qRT-PCR
		2. pNETs versus carcinoids		228	157		
Hansel [113]	12 primary pNETs	7 metastatic versus 5 nonmetastatic	Affymetrix U133A + B	65	57	IGFB3, MET	IHC
Couvelard [114]	24 well-differentiated pNETs (20 NF)	12 WDETs versus 12 WDECs	Sanger center custom 10 k	72	51	CD-34, MDR1, E-selectin, MKK4	IHC

TSGs tumor suppressor genes, *WDET* well-differentiated endocrine tumor, *WDEC* well-differentiated endocrine carcinoma, *NF* nonfunctioning

Key Research Points

- The molecular pathology of pancreatic endocrine tumors has been further investigated in the last 5 years, mostly thanks to whole genomic sequencing.
- CGH studies suggest a plausible role for a number of TSGs, which is partially confirmed by specific studies. The role of epigenetics changes, especially of methylation deserves more attention.
- A number of alterations of tyrosine kinase receptors (VEGFR), and molecular pathways (mTOR) expression and activity have been described.
- Data of microarray studies suffer of the poor heterogeneity of the samples and have not described a specific relation between expression profiles and prognosis or response to therapy.

Future Scientific Directions

- Future studies should always classify pNETs samples according to clinical and pathological standards, including WHO and TNM classification. Moreover, the tumor behavior (stable or progressive) is an issue in such an "indolent" tumor type.
- CGH array studies may help identifying putative oncogenes or TSGs.
- Microarray studies conducted in wide series of well-investigated pNETs with a relation with clinical behavior and follow-up are needed.
- More in vitro models (animal models and cell lines) are sorely needed to better understand the process of tumor growth and progression, and possibly the role of novel therapies with targeted agents.
- The relation between pNETs cells and the surrounding stroma has not been investigated and may be important, similarly to pancreatic adenocarcinoma.
- The main future focus will be the role of the immune system in pNET tumorigenesis and proliferation control, starting from PD1/PDL-1 evaluation, paving the way to immunotherapy application also in these tumors (first trials ongoing).

Clinical Implications

- Clinicians dealing with pNETs should keep in mind the possibility of inherited disorders, as the diagnostic and therapeutic strategy is different from that of sporadic cases.
- Molecular alterations may somehow predict the clinical course and possibly suggest the use of certain novel targeted therapies, such as VEGF and mTOR inhibitors.
- In this view, referral of patients to centers with more experience in clinical and molecular aspects of neuroendocrine tumors should be recommended.
- Further knowledge about molecular pathways and mutations could pave the way for new tailored target therapies.

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Abstract

Pancreatic endocrine neoplasias (pNENs) are uncommon but fascinating tumors with an annual incidence of 1 per 100,000 people. pNENs present as either functional tumors, causing specific hormonal syndromes like Zollinger-Ellison syndrome (ZES) or organic hyperinsulinism, or as pancreatic endocrine non-functional tumors (NF-pNENs). The natural history of pNENs is highly variable. Ninety percent of all insulinomas or small NF-pNENs are readily curable by surgical resection. Most other functional and late detected NF-pNENs have a less favorable chance for cure. Patients with completely resected tumors generally have a good prognosis, and an aggressive surgical approach combined with conservative treatment options in patients with advanced disease often results in long-term survival.

Keywords

Neuroendocrine tumors of the pancreas · Insulinoma · Gastrinoma · Pancreatic endocrine nonfunctional tumors · Surgical therapy

Introduction

Pancreatic endocrine neoplasias (pNENs) represent an important subset of pancreatic neoplasms (Table 1). They account for 2–4% of all clinically detected pancreatic tumors. They consist of single or multiple neoplasias and are associated in 10–20% with multiple endocrine neoplasia type 1 (MEN1). pNENs present as either functional tumors, causing specific hormonal syndromes, like Zollinger-Ellison

Table 1 Neuroendocrine neoplasias of the pancreas

Tumor (syndrome)	Incidence (%)	Presentation
Insulinoma	60–70	Weakness, sweating, tremulousness, tachycardia, anxiety, fatigue, headache, dizziness, disorientation, seizures, and unconsciousness
Gastrinoma	20–25	Intractable or recurrent peptic ulcer disease (hemorrhage, perforation), complications of peptic ulcer, diarrhea
VIPoma	4	Profuse watery diarrhea, hypotension, abdominal pain
Glucagonoma	4	Migratory, necrolytic skin rash, glossitis, stomatitis, angular cheilitis, diabetes, severe weight loss, diarrhea
Somatostatinoma	<5	Weight loss, cholelithiasis, diarrhea, neurofibromatosis
Carcinoid	<1	Flushing, sweating, diarrhea, edema, wheezing
ACTHoma	<1	Cushing's syndrome
GRFoma	<1	Acromegaly
PTH-like-oma	<1	Hypercalcemia, bone pain
Neurotensinoma	<1	Hypotension, tachycardia, malabsorption
Nonfunctional tumors	40–50	Obstructive jaundice, pancreatitis, epigastric pain, duodenal obstruction, weight loss, fatigue

syndrome or organic hyperinsulinism, or as nonfunctional pNENs with symptoms similar to pancreatic adenocarcinoma [1]. This chapter focuses on the management and surveillance of sporadic pNENs.

Epidemiology

pNENs are rare tumors but are detected more and more often [2]. They occur in approximately 1 in 100,000 people per year [3]. pNENs show no significant gender predilection and occur at all ages. Overall, the sporadic form occurs 10–20 years later than inherited pNENs in multiple endocrine neoplasia type1 (see following chapter).

Classification of pNENs

The WHO classification 2000 of neuroendocrine tumors introduced the terms "neuroendocrine tumor" and "neuroendocrine carcinoma." It aimed to separate benign from malignant tumors, introducing the concepts of benign NET, NET of unknown behavior, and malignant neuroendocrine carcinoma. The important change implied by the classification of gastrointestinal NEN 2010 was based on the introduction of the concept of grading (based on Ki-67) and staging, in analogy to other malignant neoplasias [1]. The 2010 classification also separated between so-called

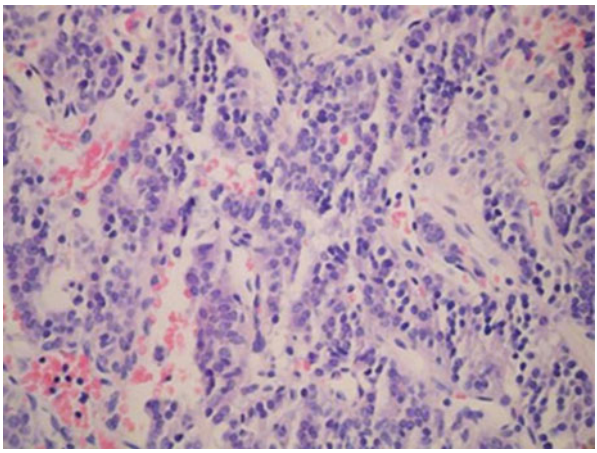

Fig. 1 H&E staining with the typical trabecular pattern of a well-differentiated pNEN

well-differentiated NET (G1 and G2) (Fig. 1) and poorly differentiated neuroendocrine carcinomas (NEC-G3) [1].

While the well-differentiated pNEN carry organ-specific somatic genetic alterations such as *MEN1*, *DAXX*, *ATRX*, *TSC*, and *NF1* mutations in, poorly differentiated NEC seem to share mutations of the non-endocrine carcinomas of the respective organs together with *p53* or *RB* mutations [4].

Imaging and Staging of pNENs

Morphological Imaging

Conventional radiological imaging modalities, such as ultrasound, CT, and MRI, are used in general for abdominal imaging and are also important in patients with pNENs. Specific protocols should be used for CT and MRI, because they are mandatory to achieve high tumor detection rates in patients with pNENs [5].

Computed Tomography

Multiphase, contrast-enhanced CT protocols are obligatory for pNEN imaging. To achieve adequate separation of the contrast phases, short scan times facilitated by multislice scanners and high-contrast agent flow rates should be used [6]. Scans before contrast which facilitate to detect calcifications, in the arterial phase with the typical pNEN enhancement and the portal venous phase to detect liver lesions, should be carried out. Because pNENs have a strictly arterial blood supply, the exact timing of the arterial phase is critical for successful pNEN imaging and should start to enhance as soon as the contrast material arrives through the arterial system. Consequently, optimal lesion to pancreas contrast can be obtained between arrival in the aorta and the pancreatic parenchymal phase [6].

Magnetic Resonance Imaging

Abdominal protocols for pNEN imaging can be derived from standard abdominal imaging protocols, including morphological T1- and T2-weighted images in different orientations. Thin slice imaging of the pancreas should be used for pNENs [2].

Molecular Imaging

The role of molecular imaging in staging, follow-up imaging, and localization of pNENs and their metastases became more important recently owing to the identification of new targets with concomitant development of respective tracers.

Somatostatin Receptor Scintigraphy and SPECT/CT

Because pNENs express somatostatin receptor (SSTR) in 80–100% of cases, somatostatin receptor scintigraphy (SRS) with ^{111}In-diethylenetriaminepentaacetic acid-D-Phe^{1}-octreotide (^{111}In-DTPA-octreotide) turns out to be an essential part of the management of patients with this type of tumor [7]. False-positive results in SRS are possible in nonneoplastic SSTR-positive tissue-like inflammatory lesions [8]. Furthermore, the sensitivity of SRS in the detection of benign insulinomas remains low with 50–60% [2].

Somatostatin Receptor PET/CT

PET imaging with ^{68}Ga-labelled somatostatin agonists provides better results than SRS and provides numerous advantages. The European Neuroendocrine Tumor Society (ENETS) guidelines recommend imaging of pNENs with SRS; nevertheless, SSTR PET/CT should be the first choice wherever available because the higher sensitivity of PET-based molecular imaging changes the management strategy in more than 70% of patients [2, 7]. One should keep in mind that a physiological tracer uptake in the uncinate process of the pancreas, adrenal glands, thyroid gland, and accessory spleen is possible and can lead to false-positive diagnosis [9].

^{18}F-Fluorodeoxyglucose (^{18}F-FDG) PET/CT

As pNENs usually do not have a high glucose turnover rate, the sensitivity of ^{18}F-FDG PET/CT is low, especially in well-differentiated NET (G1 and G2). Therefore, ^{18}F-FDG should not be used for this purpose. In contrast, high glucose metabolism is found in poorly differentiated NETs, resulting in a high tumor detection rate of ^{18}F-FDG PET/CT in G3 NECs. In consequence, negative ^{18}F-FDG PET/CT scans imply a low aggressiveness and a higher survival rate [10].

Glucagon-Like Peptide-1 Receptor (GLP-1R) Imaging

GLP-1R is overexpressed at a high incidence in almost all insulinomas. They are, therefore, an ideal target for molecular imaging [11].

Insulinomas

Insulinomas are the most frequent of all functioning pNENs. The incidence was reported to be two to four patients per million population and year. Insulinomas have been diagnosed in all age groups with a highest incidence found at age 40–60 years. Females seem to be slightly more frequently affected [12]. The etiology and pathogenesis of insulinomas are unknown. No risk factors have been associated with these tumors. Virtually all insulinomas are located in the pancreas or are directly attached to it. Tumors are equally distributed within the gland. Approximately 90% of insulinomas are solitary; the remaining 10% are multiple and are associated with MEN1 syndrome [13]. Most insulinomas are small. Forty percent are less than 1 cm in diameter, 66% are less than 1.5 cm, and 90% are less than 2 cm. Only 10% of the tumors are malignant at time of diagnosis.

Clinical Symptoms

Insulinomas are characterized by fasting hypoglycemia and neuroglycopenic symptoms, and occasionally sympathoadrenal autonomic symptoms [12, 13]. The episodic nature of the hypoglycemic attacks is due to the intermittent insulin secretion by the tumor. Most important symptoms of central nervous system dysfunction include diplopia, blurred vision, confusion, abnormal behavior, and amnesia. Some patients might develop loss of consciousness and coma or even permanent brain damage. The release of catecholamines produces symptoms such as sweating, weakness, hunger, tremor, nausea, anxiety, and palpitation. Whipple developed a symptom triad bearing his name to identify patients with insulinoma more accurately. These symptoms include signs and symptoms of hypoglycemia after fasting or exercise, blood glucose of less than 45 mg/dL when symptomatic, and symptoms relieved by intravenous or oral glucose. These symptoms usually occur when serum glucose is less than 40 mg/dL [14].

Special Diagnostic Procedures

Biochemical Testing

A fasting test that may last up to 72 h is regarded as the most sensitive test. Usually insulin, proinsulin, C-peptide, and blood glucose are measured in 1–2 h intervals to demonstrate an inappropriately high secretion of insulin in relation to blood glucose.

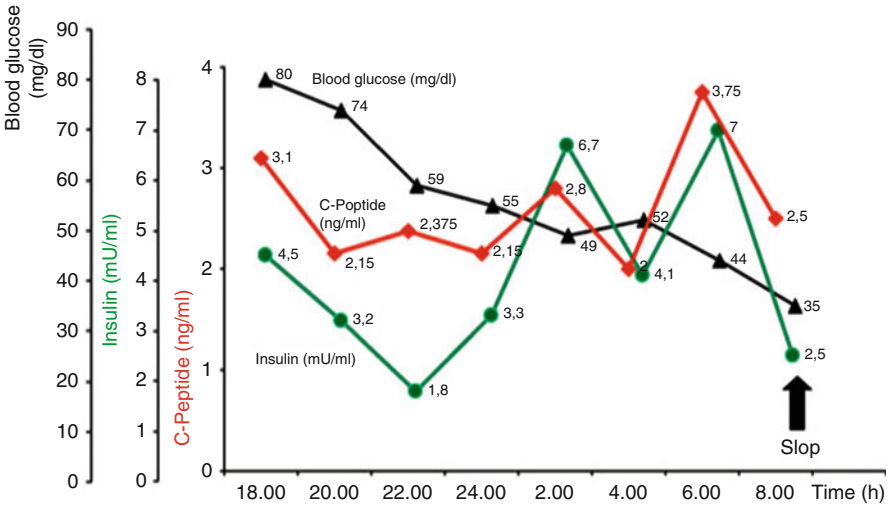

Fig. 2 Example of a typical fasting test of a patient with an insulinoma

About 80% of insulinomas are diagnosed by this test, most of them in the first 24 h [12]. In most reports, one-third of patients develop symptoms within 12 h, at least 80% within 24 h, 90% in 48 h, and 100% in 72 h [12]. Continuous C-peptide level demonstrate the endogenous secretion of insulin and exclude factitious hypoglycemia by insulin injection. An example of a fasting test is given in Fig. 2.

Treatment

Surgical cure rates in patients with the biochemical diagnosis of insulinoma range from 77% to 100% [13]. At surgical exploration, the abdomen is initially explored for evidence of metastatic disease. Then a meticulous surgical exploration should follow, i.e., an extended Kocher maneuver to be able to palpate the head, and mobilization of the distal pancreas and the spleen should follow to explore the body and tail of the gland to examine the distal pancreas carefully and completely. IOUS should then be used to confirm the presence of the insulinoma (Fig. 3) or to detect nonpalpable lesions and also to realize the relation of the tumor to the pancreatic duct. Identification of the pancreatic duct and determination of its proximity to the insulinoma can guide safe enucleation of the tumor. This approach can minimize the likelihood of a postoperative pancreatic fistula. Tumor enucleation, when feasible, is the technique of choice. If the tumor is located in the pancreatic tail, a distal spleen-preserving pancreatic resection might be the procedure of choice. To be considered malignant, these tumors must show evidence of either local invasion into surrounding soft tissue or verification of lymph node or liver metastasis. Malignant insulinomas account for only about 5–10% of all insulinomas. Aggressive attempts for resection are indicated, since there is no effective medical treatment

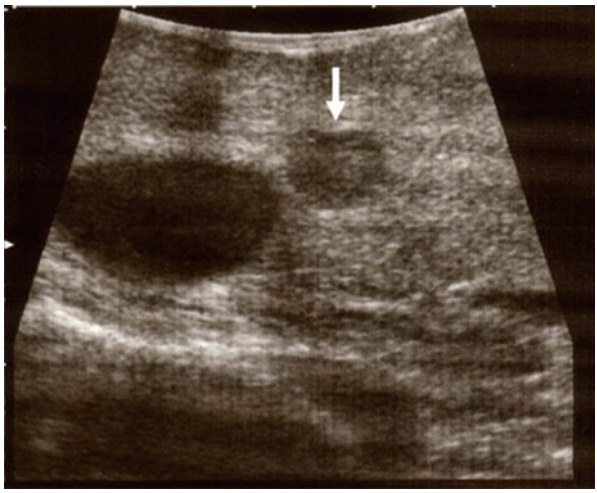

Fig. 3 Endosonography shows a typical hypoechoic insulinoma (*arrow*) in the head of the pancreas

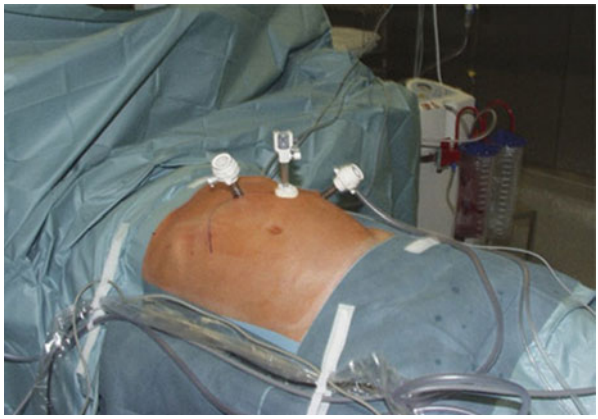

Fig. 4 Laparoscopic operation for pNENs. The patient is placed in a half-lateral decubitus position with the *left side* uppermost for tumors in the body/tail of the pancreas. The surgeon and assistant stand on the *left* of the patient, the cameraman and scrub nurse on the opposite side. Two monitors are used. Typical port sites for resection of lesions in the body/tail of the pancreas are shown

option to control hypoglycemia. Malignant insulinomas located in the body or tail of the pancreas are effectively treated by distal pancreatectomy with splenectomy and lymphadenectomy. For tumors located in the head of the gland, resection requires pancreaticoduodenectomy [15].

Recent advances in laparoscopic technique and instrumentation have enabled surgeons to approach complex procedures laparoscopically. This is also true for insulinomas [16–18], which should be favored as the procedure of choice [1]. The patient is placed in half lateral position with the left side up for tumors located in the body or tail of the pancreas, or with the right side up for tumors in the head of the gland, and in the reverse Trendelenburg position. Four 10–12 mm trocars are inserted in the abdominal wall: 3–4 cm above the umbilicus, in the xiphoid area, subcostal on the mid-axillary line, and in the subcostal midclavicular line (see Fig. 4). The pancreas is exposed after opening the lesser sac after mobilizing its head. Laparoscopic ultrasound can be used to identify nonvisible tumors and

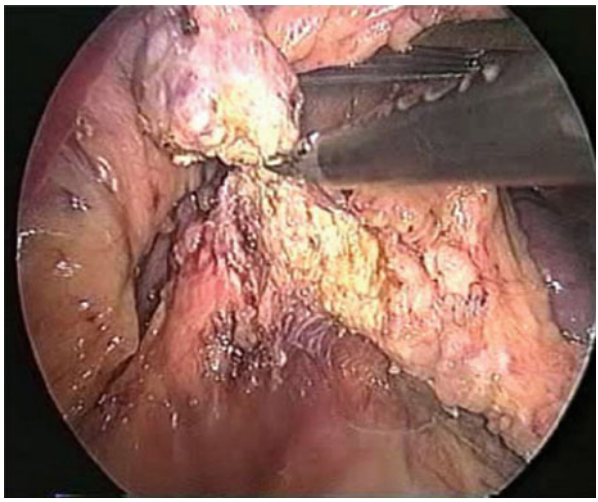

Fig. 5 Laparoscopic enucleation of an insulinoma in the pancreatic tail

determine the relationship of the lesion to surrounding veins and the pancreatic duct. Laparoscopic ultrasound can be particularly helpful in identifying lesions in the tail that are often missed by endoscopic ultrasound. For superficial ventral tumors, laparoscopic enucleation is undertaken with electrocautery or laparoscopic coagulating shears (see Fig. 5). Small pancreatic vessels can be clipped and cut. Tumors located deep in the body or tail of the pancreas and those in close proximity to the pancreatic duct require distal pancreatectomy. In cases where visualization and ultrasound fail, a hand port can be used to allow palpation of the gland. Tumors situated very distally near the splenic hilum are especially difficult to identify. It is worthwhile preserving the spleen during this procedure if it can be accomplished safely. The pancreatic tail and/or body should be meticulously dissected from the splenic vessels (Kitamura technique), or these vessels may be resected together with the pancreas, leaving the spleen vascularized by the short gastric vessels [19].

Postoperatively, blood sugar levels begin to rise in most patients within the first hours after removal of an insulinoma (reactive hyperglycemia). To preserve pancreatic function and reduce the risk of iatrogenic diabetes mellitus, patients in whom tumor localization is not successful at operation should not undergo blind resection.

Nonfunctioning Tumors

Clinically pancreatic endocrine nonfunctional tumors (NF-pNENs) produce none, or insufficient quantities of peptides, or hormones, such as pancreatic polypeptide, that do not cause any hormonal symptoms [20]. Because of modern imaging modalities, they have been diagnosed more frequently and now represent at least 50% of pNENs. At operation these tumors are generally larger than their functional counterparts and are located equally throughout the pancreas.

Clinical Symptoms

Nowadays a significant number of NF-pNENs are detected incidentally during abdominal imaging for unspecific symptoms or for reasons not attributed to the pancreas. Other patients with large tumors usually present late owing to the lack of a clinical/ hormonal marker of the tumor's activity. Therefore, in contrast to functioning pNENs, patients with NF-pNENs present either with various nonspecific symptoms, as abdominal pain, weight loss, or pancreatitis. In some cases, liver metastases are the first symptom or finding [20].

Differential Diagnosis

Because an aggressive surgical approach is justified even in locally advanced or metastatic NF-pNENs, differentiation from the more aggressive pancreatic adenocarcinomas is extremely important (Table 2).

Diagnostic Procedures

Biochemical Testing

Measurement of detectable serum or plasma levels of various hormones can establish the diagnosis of a NF-pNENs. Chromogranin A (CgA) is considered the best tumor marker currently available for the evaluation and follow-up of patients with NF-pNENs, as these tumors do not reliably produce any other suitable marker. Plasma CgA is elevated in 60% to 100% of patients with NF-pNENs. Furthermore, up to 75% of NF-pNENs are associated with increased serum levels of pancreatic polypeptide [21]. The combination of chromogranin A with measurement of pancreatic polypeptide increased the sensitivity from 84 to 96% in NF-pNENs.

Table 2 Differences between pancreatic cancer and pancreatic endocrine nonfunctional tumors (NF-pNENs)

	Pancreatic cancer	NF-pNENs
Tumor size	<5 cm	>5 cm
CT scan	Hypodensity	Hyperdensity
	No calcifications	Calcifications possible
Chromogranin A in blood	Negative	Positive
Somatostatin-receptor-scintigraphy	Negative	Positive

Treatment

According to the WHO classification, the size of the endocrine tumor correlates with malignant growth. Therefore, in localized tumors larger than 2 cm, aggressive surgery and, if required, resection of adjacent organs (stomach, colon, kidney, adrenal gland) and/or major venous resection are indicated [22]. At the present time, most would advocate an aggressive surgical approach for the management of malignant NF-pNENs even in the presence of localized metastases [15, 22]. The major goal is a potentially curative R0 resection by either partial pancreatoduodenectomy or distal splenopancreatectomy depending on the localization of the tumor. As lymph node metastases are frequently encountered, regional lymphadenectomy with en-bloc resection of the primary tumor is the goal [15, 22]. In case that the diagnosis is already made preoperatively and it is a highly proliferative (Ki67 $>$ 20%) G3 tumor, several experts would deny the indication for resection, since the prognosis is extremely poor [22]. In contrast, no data exist with respect to a positive effect of surgery on overall survival in small ($<$2 cm), possibly benign or intermediate-risk pancreatic endocrine tumors. Thus, the possibility of surgical cure has to be weighed against the operative morbidity, mortality, and long-term complications associated with pancreatic surgery [22].

Laparoscopic and Robotic Surgery for pNENs

As already mentioned, most patients with insulinomas are ideal candidates for a minimally invasive approach, because these tumors are small, solitary, and benign. The first successful laparoscopic resection was first reported by Gagner et al. in 1996 [23]. As mentioned earlier, the most sensitive method of localization is intraoperative palpation and IOUS. In laparoscopic surgery, palpation is not possible. It has been reported that preoperative localization, mainly by endoscopic ultrasonography, is crucial for the decision to operate laparoscopically [24, 25] and that minimally invasive surgery for pNENs should be undertaken only if laparoscopic ultrasound is available. Laparoscopic ultrasound helps the surgeon to decide whether to use enucleation or resection, a decision that will depend on the proximity to the main pancreatic duct or large blood vessels.

Laparoscopic enucleation is reserved for tumors less than 2 cm diameter located on, or near, the surface of the pancreas, and not in contact with splenic vessels, the portal vein or the main pancreatic duct. If these criteria are not met, laparoscopic spleen-preserving distal pancreatectomy should be the preferred choice.

MEN1 patients who have an insulinoma or small NF-pNENs can also benefit from a laparoscopic approach [26]. Gastrinomas are yet not considered candidates for a laparoscopic approach for two reasons. First, most gastrinomas are usually located in the duodenum and bidigital palpation after duodenotomy is essential to identify the small tumor. Second, most pancreatic gastrinomas are over 2 cm in diameter at diagnosis and reveal metastases in up to 70% of patients requiring a

meticulous lymphadenectomy, which is not ideal for a laparoscopic approach. The same holds true for rare functioning pNENs [15]. Given the current data, laparoscopic enucleation or resection of insulinoma and most NF-pNENs is feasible and safe, so that it might be become the future procedure of choice for insulinomas [16–18]. Nevertheless, it seems clear that such treatment should be offered only by surgeons who are experienced in both endocrine pancreatic operations and advanced laparoscopic surgery. The same is true for robotic surgery, which emerged in the last years as a new technical possibility [27, 28]. As technology advances and experience with robot-assisted surgery will increase, it is likely to become an alternative method of pancreatic resection for pNENs.

Gastrinomas (Zollinger-Ellison Syndrome)

Gastrinomas are functionally active endocrine tumors of the pancreas accounting for about 20% of pNENs, second in frequency to insulinomas. Gastrinomas were first described in 1955, when Zollinger and Ellison, of the Ohio State University Medical School, described two patients with islet cell tumors associated with atypical peptic ulceration of the jejunum [29]. Approximately 0.1% of patients with duodenal ulcers have evidence of Zollinger-Ellison syndrome. The reported incidence is between 0.5 and 4 per million of the population per year. Zollinger-Ellison syndrome is more common in males than in females, with a ratio of 3:2. The mean age at the onset of symptoms is 38 years, range 7–83 years in some series. The etiology and pathogenesis of sporadic gastrinomas are unknown. The anatomical area harboring the vast majority of these tumors encomprise the head of the pancreas, the superior and descending portion of the duodenum, and the relevant lymph nodes and has been termed the "gastrinoma triangle" (see Figs. 6 and 7) [30]. More than 90% of the duodenal gastrinomas are located in the first and second part of the duodenum and are limited to the submucosa in 54% of patients.

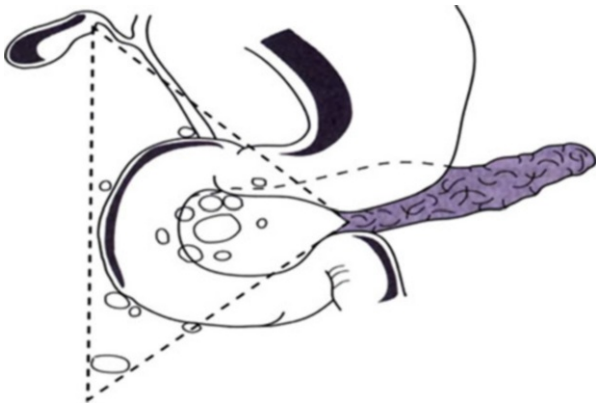

Fig. 6 Gastrinoma triangle

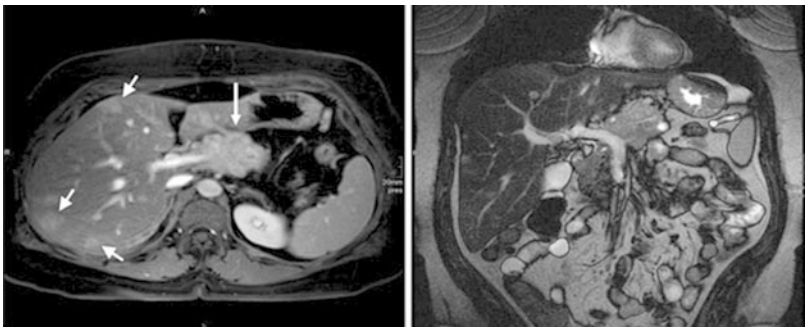

Fig. 7 Enhanced computed tomographic scan demonstrates a large pancreatic gastrinoma (*large arrow*) with diffuse liver metastases (*arrowheads*)

Clinical Symptoms

In patients with ZES, abdominal pain is the most frequent complaint either alone or with diarrhea, followed by heartburn, nausea, or bleeding. The abdominal pain is primarily due to peptic ulcer disease or gastroesophageal reflux disease (GERD) and is indistinguishable in character from that seen in ordinary ulcer patients. All of the symptoms early in the course of ZES are due to the gastric acid hypersecretion secondary to the ectopic secretion of gastrin by the tumor [31].

Differential Diagnosis

In the study of Roy et al., 164 of 168 (98%) patients with ZES were misdiagnosed before the diagnosis of ZES could be established [32]. The most common misdiagnoses were idiopathic peptic ulcer disease, chronic idiopathic diarrhea, GERD, Crohn's disease, and irritable bowel syndrome.

Hypergastrinemia can be caused by conditions other than ZES. Hypergastrinemia can be associated with increased gastric acid (e.g., retained gastric antrum, short bowel syndrome, gastric outlet obstruction) or with little or no gastric acid (e.g., pernicious anemia, chronic atrophic gastritis or vagotomy).

Diagnostic Procedures

Biochemical Testing

If the patient presents gastric pH below 4.0 and serum gastrin concentration above 1000 pg/ml (normal $<$100 pg/ml), then the diagnosis of Zollinger-Ellison is confirmed. Unfortunately, the majority (40–50%) of patients present serum

gastrin concentrations between 100 and 500 pg/ml, and in these patients a secretin test should be performed. The secretin stimulation test can differentiate between patients with ZES and those with other causes of hypergastrinemia. Patients with pernicious anemia or chronic atrophic gastritis have a lost antral gastrin release, due to their achlorhydria. In contrast to ZES, these patients can be identified by gastric pH greater than 4. The patients receive 2 μg/kg of secretin intravenously. A rise in serum gastrin by more than 200 pg/ml is typically considered positive. This test has a sensitivity and specificity of >90% for detecting gastrinomas [33].

Treatment

As with all pNENs, the only chance for cure of gastrinoma is complete surgical resection, which is achieved in 26 to 100% of patients. A study compared 160 patients with ZES undergoing resection with 35 patients who had a similar stage of disease but did not undergo surgical exploration [34]. After a follow-up of 12 years, 41% of patients were cured with surgery, and significantly more patients developed liver metastases with conservative treatment (29 vs. 5%; $P < 0.001$). Fifteen-year disease-related survival was 98% after surgery and 74% after medical treatment ($P < 0.001$). These results demonstrate that routine surgical exploration increases survival in patients with ZES by increasing disease-related survival and reducing the rate of advanced disease. Therefore, routine surgical exploration should be performed in all patients with sporadic gastrinomas without evidence of diffuse hepatic metastases and who are fit for surgery.

Duodenal Gastrinomas

Duodenotomy (DUODX) should be routinely performed for all patients with ZES. Recently, Norton and colleagues underlined the importance of DUODX in patients with ZES [35]. They performed DUODX in 79 patients, and no DUODX was performed in 64 patients. Gastrinoma was found in 98% with DUODX compared with 76% with no DUODX. They could show that the use of routine DUODX increases the short-term and long-term cure rate. Duodenal exploration is undertaken via longitudinal duodenotomy in the descending part of the duodenum. Small tumors can be identified by palpation. Duodenal tumors smaller than 5 mm can be enucleated with the overlying mucosa (see Fig. 8); larger tumors are excised with full-thickness excision of the duodenal wall. After completion of this exploration, the duodenotomy is cautiously sutured longitudinally.

Because of the high incidence of lymph node metastases associated with duodenal gastrinomas, prophylactic lymph node dissection should be done [36]. In a recent study, the distribution of lymph node metastases found at the time of operation in 38 patients with sporadic duodenal gastrinomas were analyzed by mapping their location in relation to the duodenal primary [37]. Patients who had primary duodenal tumors located above the ampulla of Vater, in general, harbored positive lymph

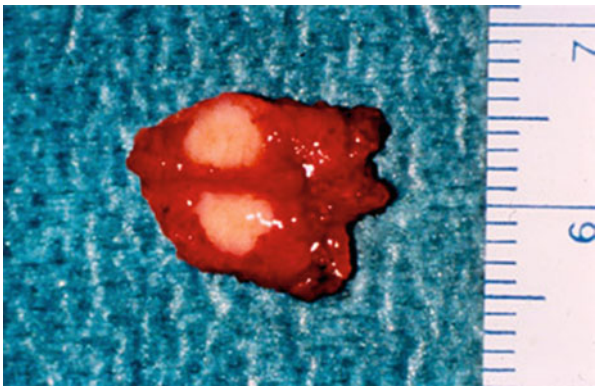

Fig. 8 Duodenal gastrinoma after duodenotomy

nodes in the superior periduodenal area, celiac axis, or periportal area. Those with primary tumors in the third and fourth portions of the duodenum had positive lymph nodes located most commonly in the superior mesenteric artery or inferior periduodenal areas. Lymph nodes were found close to the primary tumor in most cases.

Pancreatic Gastrinomas

The role of operative exploration in patients with sporadic gastrinomas is relatively well defined. Most of these non-MEN1 gastrinomas are solitary, identifiable at laparotomy, and resectable with simple enucleation. Formal pancreatic resections are typically reserved for patients with local tumor invasion. In practice, this leads to distal pancreatic resection, splenectomy, and peripancreatic lymph node dissection for gastrinomas in the pancreatic body or tail. Most of the pancreatic gastrinomas are located in the head of the gland or uncinate process. An enucleation with peripancreatic lymph node dissection is the procedure of choice in gastrinomas of the pancreatic head. For large pancreatic head gastrinomas, a pylorus-preserving pancreaticoduodenectomy is justified [15].

After removal of a gastrinoma, serum gastrin should be measured before discharge of the patient and then at 3-month intervals for the first year. Hypergastrinemia indicated residual gastrinoma tissue. A normal gastrin level may indicate a surgical cure, but a positive secretin provocative test unmasks some patients who still harbor tumor tissue.

Rare Functioning pNENs

VIPomas

Vasointestinal peptide-secreting tumors, also called VIPomas, Verner-Morrison syndrome, or watery diarrhea, hypokalemia, and acidosis (WDHA), account for fewer than 5% of islet cell tumors [1]. The two patients described by Verner and Morrison in 1958

died from dehydration and renal failure in spite of attempted intravenous hydration. The VIP directly inhibits gastric acid secretion causing achlorhydria. Sporadic VIPomas are solitary tumors, arising from the VIP-secreting cells that are usually located in the region of the pancreatic tail and body [1]. More than 60% of these tumors are malignant and metastasize to lymph nodes, liver, and bone. The secretory diarrhea ranges between 0.5 and 15 L/24 h and is usually the most prominent symptom at presentation. It results in severe loss of potassium and bicarbonate, which in turn lead to metabolic acidosis and dehydration [20]. Additional features include hypercalcemia with normal parathyroid hormone levels, hyperglycemia, and occasionally flushing of the face and the chest. The diagnosis of a VIPoma is confirmed by measurement of plasma VIP, and levels above 60 pmol/L are diagnostic.

Nearly all patients with rare functional pNENs should have abdominal exploration with the intent of complete resection of tumor. The goals of operative exploration are not only complete resection but also preparation for nonoperative management, if a complete resection is not possible. Total surgical removal of the primary tumor may be curative in approximately 60% of patients [1, 15]. In patients with metastatic VIPomas, cytoreductive debulking surgery may result in considerable palliation. The patients often require an intensive intravenous supplementation of fluid losses (often exceeding 10 l/day) and a careful correction of electrolyte and acid-base abnormalities. Somatostatin analogues reduce tumoral VIP secretion by more than 50% and inhibit intestinal water and electrolyte secretion. Via this mechanism, these drugs control the secretory diarrhea in more than 50% of patients, and significant clinical improvement is attained in another 25%. The 5-year survival rate is 60% for patients with metastases and over 90% for patients without distant metastases [1].

Glucagonomas

Glucagonomas arise from the glucagon-producing a-cells of the pancreas. Around 60% of patients already have liver metastases at the time of diagnosis [1]. Tumors that produce excessive glucagon cause a specific syndrome of diabetes mellitus, a skin rash (necrolytic migratory erythema), hypoaminoacidemia, and a tendency for deep venous thrombosis. Patients also often have stomatitis, glossitis, and cheilosis associated with the skin rash (see Fig. 9). The syndrome is diagnosed by elevated

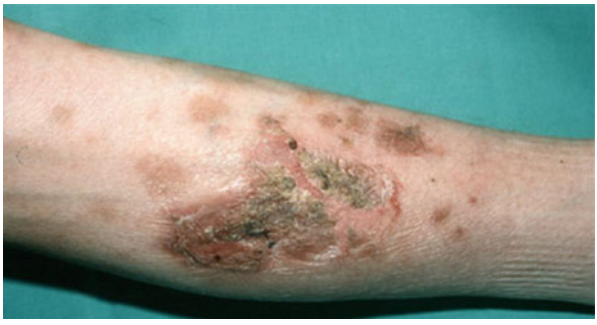

Fig. 9 Necrolytic migratory erythema in a patient with a malignant glucagonoma

plasma level of glucagon. Levels greater than 1000 pg/ml are diagnostic of the syndrome, while levels between 150 and 1000 pg/ml are suggestive. Once the syndrome is diagnosed, surgical resection of the tumor is indicated whenever possible. Preoperatively a management with somatostatin analogues and nutritional supplementation is indicated [38] to correct the nutritional deficiency and resolve the rash.

Treatment of Rare Functioning pNENs

Curative surgery is always recommended whenever feasible after careful symptomatic control of the clinical syndrome; the latter may be achieved by medical or locoregional treatments [1, 15]. Curative surgery should include an oncological pancreatic resection with lymphadenectomy. Laparoscopic resection is generally not recommended because of the need for lymphadenectomy and careful inspection for invasion/metastases.

Management of Metastases

Surgery for Liver Metastases

Liver metastases (LM) develop in 30–80% of patients with pNENs [39]. In metastatic pNENs, 5-year survival rates are around 40–60% [22]. Patients with gastrinoma and no metastatic disease have a 20-year survival rate of 95% while a 10-year survival of only 15% is reported when diffuse metastatic liver disease is present [40]. The decision for liver surgery is based on multiple factors, like tumor grading, the presence of extrahepatic distant metastases, and the presence of hormone-related symptoms [40]. Around 20–30% of patients with LM are suitable for curative intent at presentation. Cytoreductive debulking surgery in incompletely resectable metastatic disease is discussed controversially, but particularly in symptomatic patients, it may improve the quality of life [40]. For surgery with curative intent, ENETS have proposed the following criteria: (i) resectable G1/G2 liver disease with acceptable morbidity and less than 5% mortality, (ii) absence of right heart insufficiency, (iii) absence of unresectable lymph node and extra-abdominal metastases, and (iv) absence of diffuse peritoneal carcinomatosis [39]. The overall survival after hepatic resection is 46–86% at 5 years and 35–79% at 10 years in selected patients [22]. In referral centers, the 5-year survival for hepatic resections of patients with NET LM commonly exceeds 60%. Resection shows low mortality rate (0–5%) and acceptable morbidity (30%). Preselection biases due to better performance status or less advanced disease are influencing such differences in favor of the outcomes of pNEN patients undergoing surgery. Evaluation of histopathology specimens showed that often the metastatic burden in the liver is underestimated, with almost 50% of LM from NENs undetectable on preoperative imaging [22].

Ablative Therapy

Ablation of liver metastases either alone or in combination with surgical resection can be considered for appropriately selected patients [39, 41]. Image-guided ablation is an option, either alone for limited disease (tumors ideally <3 cm) or in combination with surgical resection. The lack of randomized data makes the comparison of these techniques with a surgical approach in terms of survival benefit and symptomatic relief difficult.

Liver Transplantation for Metastatic NENs

In selected cases, liver transplantation (LT) has been used to treat liver metastases from NENs. However, considerable controversy exists due to the absence of adequate available data comparing transplantation for unresectable liver metastases to other treatment modalities [42]. LT has been advocated in patients with bilateral unresectable liver metastases that are refractory to other treatments. Only a few multicenter studies and several single-center retrospective studies with small number of patients are available evaluating the survival benefits of LT for the treatment of NEN metastases [42].

Peptide-Receptor Radionuclide Therapy

In the last years, the number of pNENs that are detected is increasing. A relative new and promising therapy for patients with metastatic or non-resectable disease is peptide receptor radionuclide therapy (PRRT). The results of PRRT with ^{111}In-DTPA-octreotide were promising, whereas the number of patients with a complete or partial response was low. In the following years, radiolabelled somatostatin analogue therapy became more advanced, with the introduction of PRRT with analogues labelled with the β-emitting radionuclides lutetium-177 or yttrium-90 [43].

The efficacy of lutetium-177 in SSR-positive NENs is supported by the phase III NETTER-1 trial [44]. This trial compared ^{177}Lu delivered concurrently with standard dose (30 mg) octreotide to high dose (60 mg) octreotide LAR for patients with disease progression on standard dose octreotide. At the time of analysis, both median PFS (not yet reached vs 8.4 months, HR = 0.209; 95% CI: 0.129–0.388; $p < 0.0001$) and OS (22 vs. 13 months; $p < 0.0186$) were significantly improved for patients on the ^{177}Lu arm.

For pNENs, the possibility of a neoadjuvant PRRT was evaluated in the very last years. There is not much experience with this specific indication for PRRT. However, the few case reports available show promising results, even in patients with limited metastatic disease. Data from Van Vliet et al. showed successful surgery in 9 of 29 patients treated with neoadjuvant PRRT [45].

Biotherapy

Somatostatin Analogues (SSAs) and Interferon

Very recently, the phase III placebo-controlled CLARINET trial expanded the role of SSAs for tumor control in NEN [46]. In this study, over 200 patients with well- or moderately differentiated, nonfunctioning, SSTR-positive NENs with a Ki-67 of <10% were randomized to receive either lanreotide 120 mg every 4 weeks or placebo. Lanreotide was associated with a significant prolongation of PFS, with a median not reached versus a median of 18 months in the placebo arm (hazard ratio (HR) 0.47; $p < 0.001$). The estimated rates of PFS at 24 months were 65.1% in the lanreotide group and 33% in the placebo group. The benefit in the patients with midgut NET (HR 0.35; $p = 0.009$) was greater than in the pancreatic subset (HR 0.58; $p = 0.06$).

SSAs in Insulinomas

Most insulinomas are benign and can be cured by surgery. In the rare metastasizing insulinomas, SSA treatment often is of limited value for glycemic control. One probable reason is the low expression of SSTR2 [47]. Further studies are needed to evaluate the potential role of pasireotide in patients with malignant insulinoma. Pasireotide does not inhibit counter-regulatory glucagon secretion and often induces hyperglycemia. Therefore, it could be helpful for treating hypoglycemia in insulinoma patients.

SSAs in Zollinger-Ellison Syndrome

SSA lowers gastrin levels and can ameliorate symptoms of Zollinger-Ellison syndrome. However, proton pump inhibitors are the treatment of choice for symptom control as they are highly effective and oral available [47].

SSAs in Verner Morrison Syndrome

Treatment with SSAs results in a rapid reduction of the excessive secretory diarrhea caused by vasoactive intestinal polypeptide (VIP) secreting pNENs and is indicated in this disease.

SSAs in Glucagonoma Syndrome

The necrolytic migratory erythema – a characteristic skin rash caused by glucagon secreting pancreatic NETs – can resolve rapidly after initiation of SSA treatment. The European Neuroendocrine Tumour Society (ENETS) therefore recommends treatment with SSA in patients with glucagonoma syndrome [47].

Interferon

According to the ENETS guidelines, IFN-α can be considered for symptomatic treatment of functional pNENs [47] in case of intolerance of SSAs and insufficient

antisecretory effects of SSA. However, due to the unfavorable toxicity profile, IFN-α is not first therapeutic choice in pNENs.

Novel Targeted Drugs

Novel targeted drugs (everolimus and sunitinib) are approved for pNENs based on the results of two placebo-controlled trials on progressive pNENs [48, 49]. The median PFS is around 11 months with either of the drugs, while tumor remission occurs in 5% and <10% of the patients with everolimus and sunitinib, respectively [39]. The use of either everolimus or sunitinib is recommended in progressive G1/G2 pancreatic NET, irrespective of Ki-67 and tumor burden. While comparative data of both drugs are lacking, the selection of the targeted drug is based on the medical history of the patient, the side effect profile of the drug and accessibility to the treatment.

Chemotherapy

Chemotherapy is one of different treatment options in pNENs and can be used in G1 or G2 neoplasias. Cytotoxic therapy combinations include: streptozotocin/5-FU or doxorubicin with streptozotocin as an alternative option. Usually, patients with pNENs with Ki-67 of 5–20% can be treated with chemotherapy. Other factors that favor chemotherapy compared to targeted drugs include bulky disease, a symptomatic patient, rapid tumor progression in ≤6–12 months, and patients with a possible chance of achieving a response to allow for surgery [39].

In NEC G3 patients, a cisplatin-based chemotherapy (e.g., cisplatin/etoposide) is considered as standard therapy and recommended as a first-line therapy (Pavel 2016). Although objective remission rates are high (40–67%), the median PFS is limited with 4–6 months [50]. Second-line systemic therapy options include FOLFOX and FOLFIRI [39].

Conclusion

PNENs are rare but fascinating tumors. Biochemical diagnosis justifies laparotomy in patients with insulinomas and gastrinomas, even if a tumor is not detected preoperatively. Whereas patients with insulinomas are usually cured, also in patients with gastrinoma, a significant surgical cure rate can be achieved. The prognosis of pNENs is much better than that of pancreatic adenocarcinoma, even though patients are frequently diagnosed with metastatic disease. Therefore, an aggressive surgical approach leads to long-term survival even in patients with malignant PETs. Although long-term cure can only be realized in a proportion of patients, significant long-term palliation can be achieved.

Cross-References

- ▶ Inherited Pancreatic Endocrine Tumors
- ▶ Laparoscopic Surgery for Pancreatic Neoplasms
- ▶ Molecular Pathology of Pancreatic Endocrine Tumors

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Molecular Pathology of Carcinomas of the Ampullary/Periampullary Region

Lena Haeberle, Jasmin Riemer, and Irene Esposito

Contents

Abstract

The ampullary/periampullary region is a complex anatomical environment giving rise to a number of heterogeneous malignancies. Ampullary carcinomas should be distinguished from periampullary duodenal, biliary, and pancreatic adenocarcinomas. A meticulous classification of periampullary/ampullary carcinomas is of great importance, as the biological behavior of the various types of carcinomas differs significantly, affecting their prognosis and therefore their clinical

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management. Subtypes of ampullary carcinomas, namely, intra-ampullary, ampullary ductal, periampullary duodenal, and ampullary NOS (not otherwise specified) carcinomas, have been recently proposed based on a detailed assessment of their gross appearance in correlation with microscopic findings. Moreover, ampullary carcinomas can be further classified as intestinal type, pancreatobiliary type, or mixed type based on the tumor's histomorphology and immunohistochemical profile.

In recent times, crucial advances have been made in characterizing carcinomas of the ampullary/periampullary region on a molecular level. Several molecular patterns seem to correlate with prognosis. Moreover, some molecular pathways, e.g., the *WNT* pathway, represent potential therapeutic targets to be used in the context of personalized medicine in the future. Gene panel analysis is a promising approach that could be used to translate these findings into clinical applications.

Keywords
Periampullary cancer · Ampullary cancer · Duodenal cancer · Distal bile duct cancer · Precursor lesions · Molecular pathology · Next-generation sequencing · NGS

Introduction

The ampulla of Vater is a small but complex anatomical landmark. It is formed by the common bile duct and the pancreatic duct, which converge to create a short common channel that drains through the papilla of Vater located in the wall of the second part of the duodenum. However, the pancreatobiliary duct system can be subject to a number of anatomical variants (Fig. 1A). For example, the common channel created by the common bile duct and pancreatic duct often does not represent a "true ampulla," which has been defined as a dilated reservoir [1]. Moreover, the length of the common channel can vary greatly. In many patients, a common channel is completely missing and the common bile duct and the pancreatic duct drain independently into the duodenum.

Anatomical regions that harbor different types of epithelia are often relevant in carcinogenesis. In the ampullary/periampullary region, the pancreatobiliary epithelium of the common bile duct, pancreatic duct, and common channel merge into the intestinal epithelium of the duodenum. This is thought to be the reason why the ampullary/periampullary region represents a hot spot for cancers of the small intestine, together with the fact that this region is also exposed to biliary juice, pancreatic juice, and duodenal juice [2].

The term "periampullary cancer" refers to neoplasms originating from four different anatomic locations within 2 cm of the major papilla of Vater (Fig. 1B) [3, 4]: (1) adenocarcinoma of the head of the pancreas, (2) ampullary cancer, (3) duodenal cancer, and (4) distal bile duct cancer (Table 1). Obstructive jaundice is a common symptom of cancers located in the vicinity of the ampulla of Vater [5].

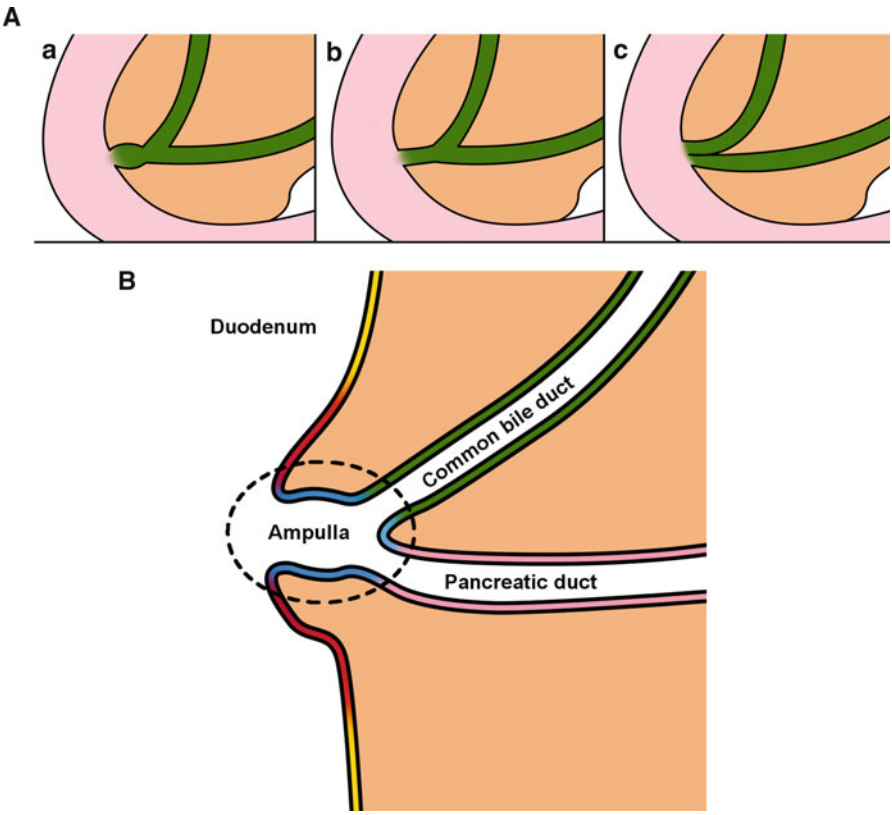

Fig. 1 (**A**) Anatomical variants of the Vaterian system: (*a*) common bile duct and pancreatic duct converge to form a dilated reservoir ("true ampulla"), (*b*) common bile duct and pancreatic duct converge to form a common channel that does not represent a true ampulla and can vary in length, and (*c*) common bile duct and pancreatic duct do not converge and drain into the duodenum independently. (**B**) Overview of the different locations of cancers of the ampullary/periampullary region: extra-ampullary duodenal carcinoma (*yellow*), periampullary duodenal carcinoma (*red*), ampullary carcinoma (*blue*), distal bile duct carcinoma (*green*), and pancreatic ductal adenocarcinoma (*pink*) (Modified from Refs. [4, 6])

Table 1 Proportion of the different tumor types among resected cancers of the ampullary/periampullary region [7–9]

Cancer type	Proportion of resected cancers (%)
Ampullary carcinoma	15–25
Duodenal carcinoma	4–9
Distal bile duct carcinoma	9–15
Pancreatic carcinoma	56–66

Other very rare tumor entities, which may also be found in the periampullary region, include duodenal neuroendocrine neoplasms, lymphomas, gastrointestinal stromal tumors (GIST), and hamartomas in patients with Peutz-Jeghers syndrome.

Distinguishing between pancreatic, ampullary, duodenal, and bile duct origin of ampullary/periampullary carcinomas can be difficult due to overlapping histopathological characteristics [4]. However, determining the exact origin is of great prognostic relevance. Survival rates are, in general, greatest for duodenal and ampullary cancers, intermediate for distal biliary cancers, and lowest for pancreatic cancer [3, 4, 7]. Although a Whipple procedure or its variants are the therapy of choice for all four cancer entities, discrepancies in survival among cancers of this region remain even after radical resection [3, 4, 8].

Several explanations exist for these differences in survival [5]: Early diagnosis due to early symptoms in small tumors arising directly from the ampulla or the periampullary duodenum compared to the usually late diagnosis of pancreatic cancers, in which biliary obstruction is often a sign of advanced disease, is one point to be considered [4]. However, there are also differences in both macroscopic and microscopic growth patterns between the tumor types, reflecting a different biological behavior: for example, ampullary and duodenal cancer generally show less vascular and perineural invasion compared to pancreatic cancer and distal bile duct cancer, which are both characterized by a highly invasive growth pattern [4, 5]. However, recent data suggest that, once adjusted for survival-determining factors like tumor size or lymph node invasion, the overall survival of patients depends on the histological type of the tumor (i.e., intestinal vs. pancreatobiliary) and not on its anatomical origin [10].

Another important aspect, which has become evident from recent studies, is that a different molecular pathogenesis affects the different biological behavior of cancers arising in the ampullary/periampullary region.

The aim of this article is to give an overview of the available data on the molecular pathology of non-pancreatic cancers of the ampullary/periampullary region. The increasing knowledge of the molecular alterations responsible for the different biological behavior of these tumors can lead to improvements in diagnosis and tumor-type-specific therapy in the future.

Sporadic Cancers of the Ampullary/Periampullary Region

A careful gross assessment of pancreatoduodenectomy specimens of patients with neoplasms of the ampullary/periampullary region represents a fundamental step and a prerequisite for the further histopathological and molecular characterization. Standard protocols that take into account the complex anatomy of such resection specimens, as well as the growth characteristics of pancreatic ductal adenocarcinoma, have been developed [11, 12] and can be applied to other neoplasms arising in the ampullary/periampullary region, which are resected according to the same surgical procedure as ductal adenocarcinoma of the pancreatic head [13].

Ampullary Cancer

Ampullary cancer is defined as a neoplasm involving the ampulla of Vater (if anatomically present), the major duodenal papilla and/or the distal ends of the common bile duct, and the main pancreatic duct, as well as their opening on the duodenal surface (Fig. 1B) [14].

Gross Appearance and Histopathological Classification

Carcinomas of the ampulla of Vater are often diagnosed when they are still relatively small, because they can lead to biliary obstruction and consecutive jaundice at an early stage (Fig. 2). Traditionally, ampullary carcinomas were classified as polypoid or ulcerating, with ulcerating carcinomas harboring a poorer prognosis [2]. In a more meticulous approach, ampullary carcinomas can be classified into four subtypes based on the site from which the tumor is thought to arise in correlation with microscopic findings regarding preinvasive and invasive components [14]: (1) *intra-ampullary* carcinomas, which show significant preinvasive exophytic components within the common channel, but no involvement of the duodenal surface; (2) *ampullary ductal* carcinomas, which are characterized by invasive components on

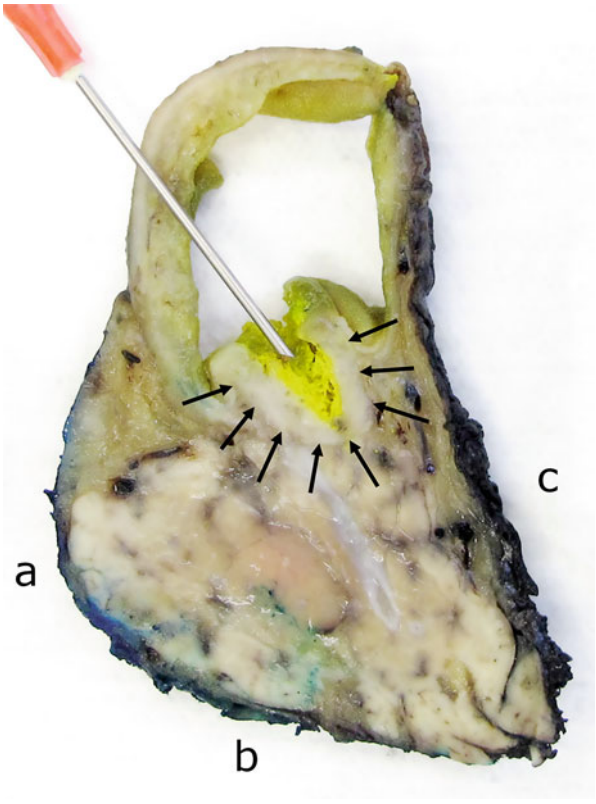

Fig. 2 Macroscopic section of an ampullary adenocarcinoma (*arrows*). The papilla of Vater was inked *yellow*, the anterior free surface of the pancreatic head was inked *blue* (*a*), the medial (superior mesenteric artery/uncinatus) margin was inked *green* (*b*), and the posterior margin was inked *black* (*c*)

the distal walls of the common bile duct and/or pancreatic duct without any significant preinvasive growth; (3) *periampullary duodenal* carcinomas, which grow into the duodenal lumen and involve the duodenal surfaces, but rarely the lumen of the ampullary common channel; and, lastly, (4) *ampullary NOS* (not otherwise specified) carcinomas, which are located at the papilla of Vater, but do not specifically fit into one of the three previous categories.

This classification is of prognostic significance. Intra-ampullary carcinomas showed the best prognosis (3-year survival 73%), while the prognosis of periampullary duodenal carcinomas was not quite as good (3-year survival 69%) and ampullary ductal carcinomas displayed the poorest prognosis (3-year survival 41%) [14]. However, this classification is not of widespread use yet; in particular, periampullary duodenal carcinoma could be still considered a separated entity and will be discussed in the section "Duodenal Cancer."

A reproducible classification of ampullary carcinomas into histological subtypes remains challenging [4]. In 1994, Kimura et al. distinguished for the first time between two histological types of ampullary carcinomas, an intestinal type and a pancreatobiliary type (Fig. 3) [15]. These were later defined as the two main types of ampullary cancer, while other rare types, like mixed-type, signet ring cell, or clear cell carcinoma, were also described [16].

Ampullary carcinomas with intestinal differentiation resemble colorectal carcinomas. They are characterized by the presence of goblets cells and frequently display adenomatous components within the tumor [4, 5]. Ampullary carcinomas of the pancreatobiliary type more closely resemble carcinomas of the pancreas or extrahepatic bile ducts [2, 4].

Determining the histological type of ampullary carcinomas is of great significance, because it acts as an independent predictor of survival: Ampullary carcinomas of the intestinal type show a significantly better prognosis than carcinomas of the pancreatobiliary type [15, 17].

The immunohistochemical analysis of selected mucins (MUC) has successfully been used to achieve a more reliable differentiation between intestinal-type and pancreatobiliary-type ampullary carcinomas [18]. Ampullary carcinomas can be classified as intestinal type either by positivity for CK20, CDX2, or MUC2 and negative staining for MUC1 or by positivity for CK20, CDX2, and MUC2, irrespective of the MUC1 staining result, while pancreatobiliary-type ampullary carcinoma can be defined as an ampullary carcinoma staining positive for MUC1 and negative for CDX2 and MUC2, irrespective of the CK20 staining result (Fig. 3) [18]. CK7 has been used to differentiate between intestinal-type and pancreatobiliary-type ampullary carcinomas in the past and was thought to be negative in the intestinal type and positive in the pancreatobiliary type, but it has not been proven as a reliable marker [18].

Precursor Lesions

In analogy to pancreatic and biliary intraductal papillary and tubular neoplasms, the term "intra-ampullary papillary-tubular neoplasms" (IAPN) has been proposed for tumor-forming preinvasive precursor lesions of the periampullary/ampullary region

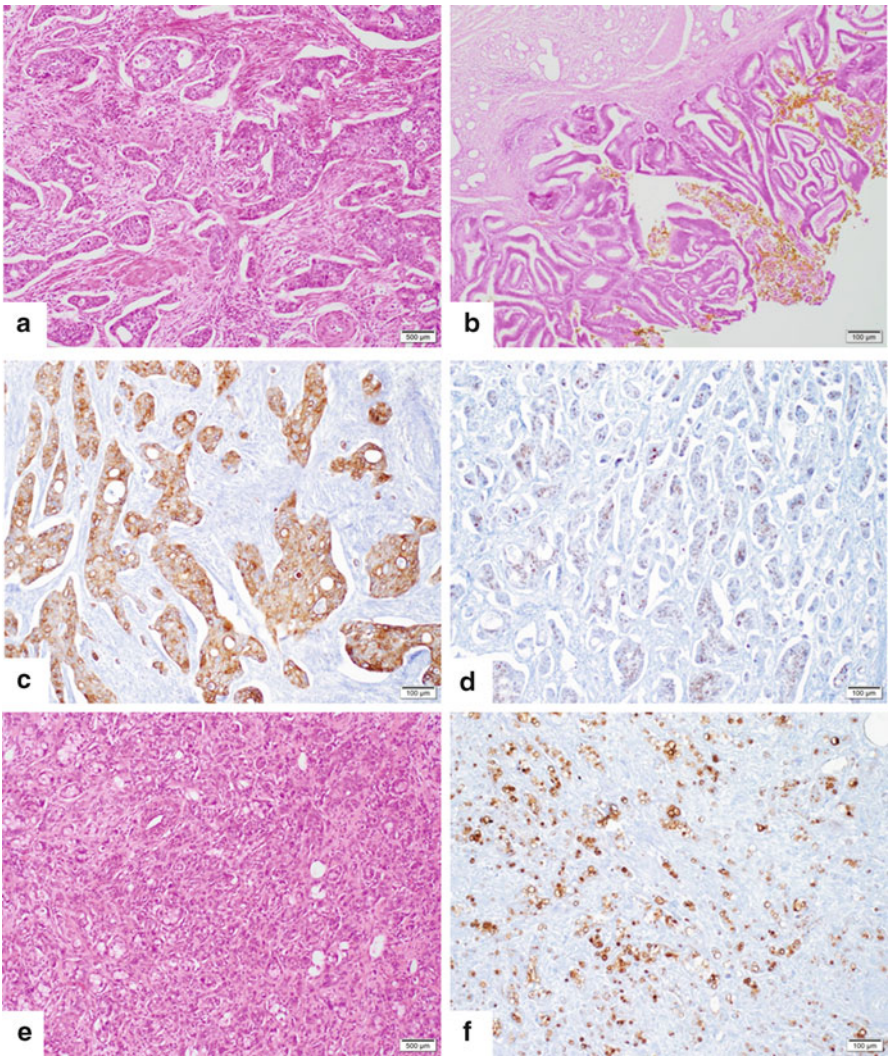

Fig. 3 Ampullary carcinoma, intestinal type (**a**–**d**) and pancreatobiliary type (**e**, **f**). (**a**) HE staining of ampullary carcinoma, intestinal type. (**b**) Intra-ampullary papillary-tubular neoplasm (*IAPN*) as a precursor lesion of ampullary carcinomas. (**c**) Positive immunostaining for CK20 in ampullary carcinoma of intestinal type. (**d**) Positive nuclear immunostaining for CDX2 in ampullary carcinoma of intestinal type. (**e**) HE staining of ampullary carcinoma, pancreatobiliary type. (**f**) Positive immunostaining for MUC-1 in ampullary carcinoma of pancreatobiliary type

(Fig. 3) [14, 19]. IAPN can show papillary and/or tubular growth patterns, low- or high-grade dysplasia, and different cell lineage morphologies (intestinal vs. gastric/pancreatobiliary) [14, 19]. Additional, non-papillary precursors seem to exist, but they have not been well characterized as a separate entity so far.

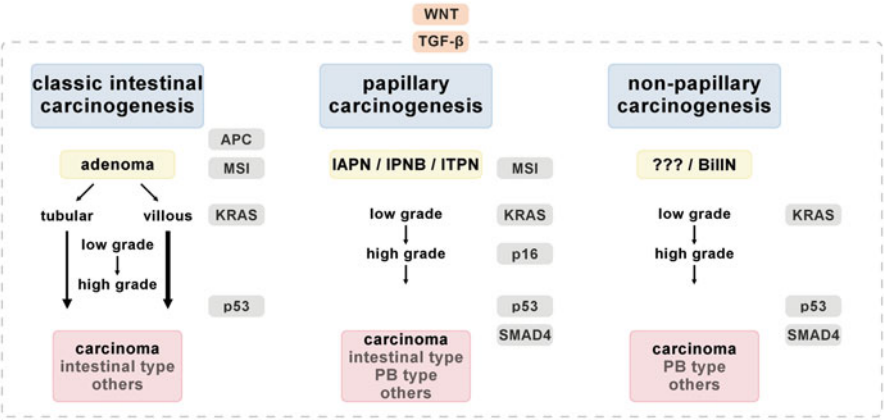

Fig. 4 Molecular pathological concept of carcinogenesis in the ampullary/periampullary region: While extra- and periampullary duodenal carcinomas most often develop from adenomas in a classical adenoma-carcinoma sequence (*left*), many carcinomas of the Vaterian system rise from papillary, tumor-forming precursor lesions like IAPN (intra-ampullary papillary-tubular neoplasms) or IPNB/ITPN (intraductal papillary neoplasms of the bile duct/intraductal tubulo-papillary neoplasms of the bile duct) (*middle*). The non-tumor-forming precursor lesions of the biliary tract are called BilIN (biliary intraepithelial neoplasia), whereas similar "flat" precursor lesions of the ampulla have not been well characterized yet (*right*). An important role of the *WNT* and TGF-β pathways has recently been described in different subtypes of cancers of the ampullary/periampullary region

Molecular Pathology

Recent advances have been made in the molecular characterization of ampullary carcinomas (Fig. 4).

Some mutations, which are shared with pancreatic ductal adenocarcinoma (PDAC) and colorectal cancer (CRC), were described in ampullary cancer over a decade ago: For example, *KRAS* mutations are detected in all three types of cancer, although the frequency varies significantly (>90% in PDAC, 31% in CRC, 37% in ampullary carcinomas) [20–22]. Mutations in tumor suppressor genes like *TP53* and *SMAD4* have been found to occur in PDAC and ampullary carcinoma in a similar frequency [5, 23]. A loss of mismatch repair proteins and a microsatellite instability, as known in the context of CRC, have also been described in ampullary carcinomas (Fig. 5) [24].

In a recent study by Gingras et al., the molecular profiles of duodenal carcinomas, bile duct carcinomas, and ampullary carcinomas were compared [25]. Some common mutations, like *KRAS*, *SMAD4*, and *TP53* mutations, as well as a high rate of microsatellite instability, could be confirmed. In addition, alterations in the *WNT* signaling pathway were found in all three cancer types and, overall, in almost half of the patients, independently from the tumor subtype. *WNT* signaling pathway disruption was found in 49% of ampullary carcinomas, and while it was more frequent in the intestinal subtype (67%), it was found in 30% of the ampullary carcinomas of the

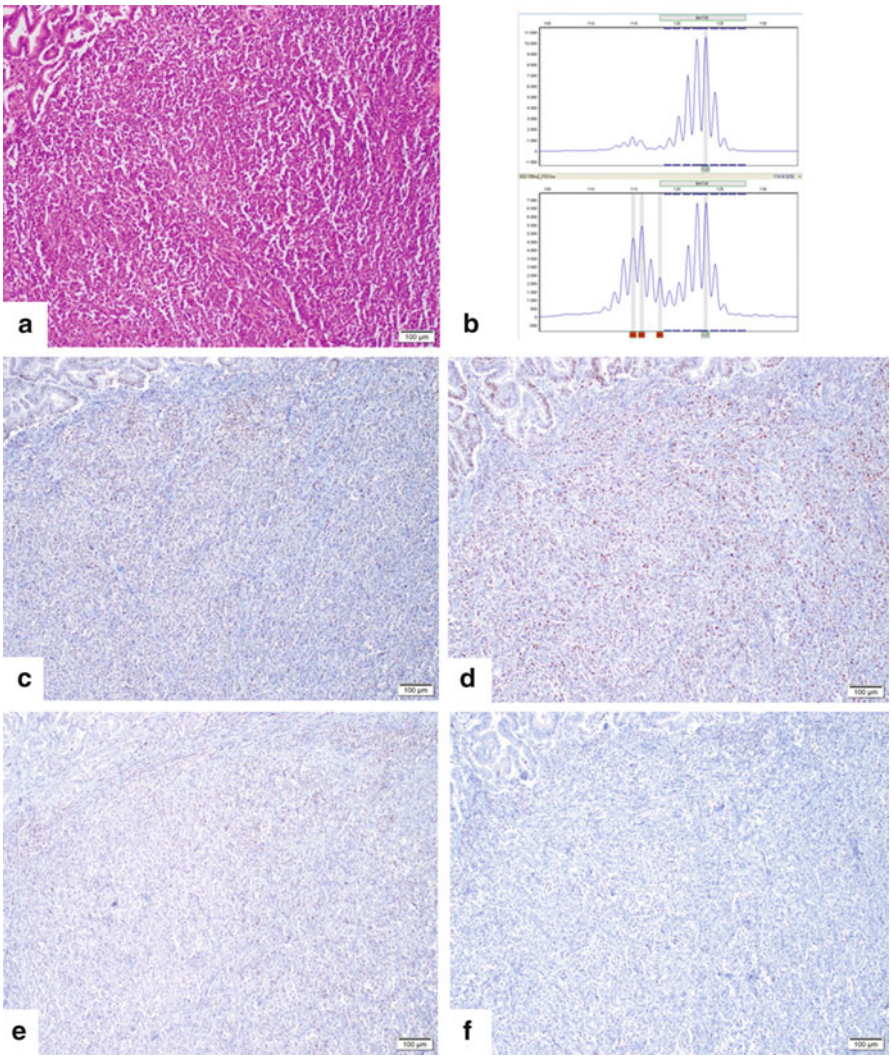

Fig. 5 Loss of mismatch repair proteins and microsatellite instability in ampullary carcinoma. (**a**) Well-differentiated glandular (*upper left*) and poorly differentiated solid pattern with many infiltrating lymphocytes in ampullary carcinoma (HE). (**b**) *BAT25* microsatellite fragment analysis. (**c**–**f**) Immunostaining for mismatch repair proteins: positive nuclear staining in 80% for *MSH2* (**c**) and *MSH6* (**d**) and negative nuclear staining for *PMS2* (**e**) and *MLH1* (**f**)

pancreatobiliary subtype as well. Interestingly, inactivating mutations of *ELF3*, a transcriptional regulator in the TGF-β pathway, were found in 11% of all carcinomas investigated in the study.

Some of the molecular alterations found in ampullary carcinomas act as prognostic factors. Microsatellite instability appears to be associated with a survival

advantage similar to what has been observed in colorectal carcinomas [25]. Mutations in the TGF-β pathway (e.g., alterations of *TGFBR2*, *ACVR1B*, *ELF3*, *SMAD4*) also seem to be associated with a better survival [25].

Mutations found in ampullary carcinomas also represent potential therapeutic targets. For example, several molecules targeting the *WNT* signaling pathway are currently being developed. The molecular status of an individual patient's ampullary carcinoma will therefore be of great interest in the future in order to perform successful personalized medicine. Thus, using next-generation sequencing (NGS) to assess ampullary carcinomas for mutations in a large panel of cancer-associated genes seems like a promising approach, especially because this can be achieved using material from EUS-guided fine needle aspiration and is therefore an option even if surgical specimens cannot be obtained [26].

Duodenal Cancer

The duodenum is the most common site of origin of adenocarcinomas of the small bowel, which altogether represent a quite rare tumor entity, accounting for less than 2% of all gastrointestinal cancers [27]. The second portion of the duodenum, including the periampullary region, represents the most common localization of small bowel adenocarcinomas with a rate of about 80% [28]. It is therefore important to distinguish between periampullary duodenal carcinomas, one of the four subgroups of cancers of the ampullary/periampullary region, and carcinomas elsewhere in the duodenum (extra-ampullary duodenal carcinomas) (Fig. 1b).

Gross Appearance and Histopathology

Macroscopically, most duodenal adenocarcinomas are relatively circumscribed with a polypoid configuration and central ulceration. Periampullary duodenal carcinomas usually do not involve much of the ampullary common channel itself, but rather grow on the duodenal surface of the papilla.

In analogy to the other cancers of this region, periampullary duodenal carcinomas are histopathologically classified as intestinal type, pancreatobiliary type, or mixed type based on their histomorphological appearance and their immunophenotype. Most often (75%) periampullary duodenal adenocarcinomas are of the intestinal type [14]. Extra-ampullary adenocarcinomas of the duodenum can histologically be distinguished in a gastric, intestinal, pancreatobiliary, and indeterminate subtype [28]. The gastric and intestinal subtypes are the most common (50% and 37%, respectively), while the pancreatobiliary type is very rare in this entity [29].

Precursor Lesions

The precursor lesions of extra-ampullary duodenal carcinomas are often referred to as adenomas in analogy to the precursors in the colorectum and are classified in tubular, tubulo-villous, and villous adenomas (Fig. 5). An adenoma-carcinoma sequence is widely accepted for small bowel adenocarcinomas as well [2].

Molecular Pathology

According to the classical intestinal adenoma-carcinoma sequence, microsatellite instability and mutations of *APC*, *KRAS*, *TP53*, and β-catenin have been described in duodenal cancers [30, 31]. While alterations in *KRAS* and *TP53* seem to occur in a similar frequency as in CRC (43% and 42%, respectively) [30], *APC* mutations are quite infrequent (0–18%) [31], suggesting some differences between the colorectal and the small bowel adenoma-carcinoma sequence.

In the previously mentioned study by Gingras et al., duodenal carcinomas were tested for the same molecular alterations as ampullary carcinomas. Interestingly, *ELF3* mutations and *WNT* signaling disruption were also found in duodenal carcinomas and were more frequent than in other cancers of the ampullary/periampullary region (72% vs. 49%) [25].

Similar to what has been observed for CRC, microsatellite instability is a positive prognostic factor in small bowel adenocarcinomas [30]. Both microsatellite instability and mutations in the TGF-β pathway (see section about ampullary cancer) seem to be associated with a better survival [25].

Distal Bile Duct Cancer

Bile duct cancer (cholangiocarcinoma) is a rare, heterogeneous entity that represents only 3% of all gastrointestinal malignancies [32]. It can be classified according to its anatomical site as peripheral intrahepatic, (peri)hilar, or extrahepatic [33].

Gross Appearance and Histopathology

Distal extrahepatic bile duct cancer can involve the ampullary/periampullary region and therefore be misdiagnosed as ampullary cancer (Fig. 1b).

Histopathologically, distal bile duct cancers are adenocarcinomas with a tubular or tubulo-papillary growth pattern and mostly a pancreatobiliary differentiation with expression of CK7 and CK19, MUC1, BER-EP4, and CEA at immunohistochemistry [33]. Gastric, intestinal, mucinous (colloid) variants, as well as undifferentiated subtypes, have been described as well.

Precursor Lesions

There are three distinct types of precursor lesions of distal bile duct adenocarcinoma: biliary intraepithelial neoplasia (BilIN), intraductal papillary neoplasms (IPN or IPNB), and intraductal tubulo-papillary neoplasms (ITPN) (Fig. 5) [34–36].

BilIN are non-tumor-forming, flat precursor lesions in analogy to PanIN of the pancreas. Molecular alterations of BilIN include *KRAS* mutations, which increase in frequency during progression from low-grade to high-grade BilIN, as well as *p53* overexpression, loss of *SMAD4*, p16 inactivation, and an altered expression of p21 and cyclin D1 [34].

IPNB/ITPN are tumor-forming papillary precursor lesions of distal bile duct carcinomas. In analogy to intraductal papillary mucinous neoplasms (IPMN) of the

pancreas, IPNB can be classified as intestinal, pancreatobiliary, gastric, or oncocytic subtype [35]. A stepwise progression from IPNB to invasive adenocarcinoma is assumed [35]. Important molecular alterations that occur during the progression from IPNB to invasive carcinoma include *KRAS*, *TP53*, and p16 mutations as early events and loss of *SMAD4* as a late event [35]. In contrast, molecular alterations frequently observed in ITPN include p16 and *TP53*, while mutations of *KRAS* and *PIK3CA* and loss of *SMAD4* seem to be rare events [36].

Molecular Pathology

NGS has been used in recent studies in order to shed light on the molecular alterations underlying the carcinogenesis of the biliary tree. These studies unraveled a number of novel molecular alterations in biliary carcinomas in general, as well as significant differences in the molecular pathology of intrahepatic and extrahepatic bile duct carcinomas. While, for example, *IDH1*/*IDH2* mutations seemed to be restricted to intrahepatic biliary carcinomas, genetic alterations of *ERBB2* were more frequently found in extrahepatic biliary carcinomas [37, 38]. *TP53* and *KRAS* are the most frequently observed mutated genes in extrahepatic bile duct cancer (45% and 40%, respectively) [37]. Other molecular alterations frequently found in extrahepatic bile duct carcinoma include mutations of *SMAD4*, *FBXW7*, *CDKN2A*, and *CDKN2B* or aberrations in the MAPK, mTOR, and DNA repair pathway [37]. Alterations of molecules of the MAPK and mTOR pathways can be used as druggable targets in new approaches of personalized molecular therapy [38].

As mentioned above, a recent study by Gingras and colleagues found *ELF3* and *WNT* pathway alterations also in distal bile duct cancers. However, alterations of the *WNT* pathway were found significantly less frequently in distal bile duct cancers than in ampullary cancers and duodenal cancers (30% vs. 49% vs. 72%) [25].

TP53 and *KRAS* mutations were both found to be negative prognostic factor in bile duct carcinomas, with *TP53* being an independent prognostic factor at multivariate analysis [38].

Alterations in the chromatin-modulating genes *BAP1* and *PBRM1* were also associated with a poor prognosis in extrahepatic bile duct carcinomas [37].

Cancers of the Ampullary/Periampullary Region in Hereditary Cancer Syndromes

Familial Adenomatous Polyposis (FAP)

Familial adenomatous polyposis (FAP) is a rare autosomal dominant disease caused by an inactivating germline mutation in the adenomatous polyposis coli (*APC*) gene on the long arm of chromosome 5 [4]. *APC* is a tumor suppressor protein in the *WNT* signaling pathway and is part of a complex that prevents the nuclear translocation of the transcription factor β-catenin. Extensive colorectal polyposis is pathognomonic for FAP. Patients with FAP have a lifetime risk of up to 100% for CRC if they are not treated by prophylactic proctocolectomy. The majority of all FAP patients will

develop duodenal polyps, normally with a slow progression and an overall risk of duodenal cancer of 5% (100- to 330-fold higher compared to the general population) [39–41]. Duodenal adenocarcinoma, preferentially located in the periampullary region, and ampullary carcinoma are two of the most common causes of death in FAP patients (approximately 3%) [42].

In analogy to sporadic tumors, an adenoma-carcinoma sequence is well established for duodenal/ampullary carcinomas in FAP patients. FAP patients may additionally develop dysplastic lesions in the biliary tree and bile duct cancer.

Ampullary and Duodenal Cancers in Hereditary Nonpolyposis Colorectal Cancer (HNPCC)

Lynch syndrome is an autosomal dominant genetic condition with incomplete penetrance and represents the most common inherited colon cancer syndrome [4]. The underlying molecular alterations in patients with Lynch syndrome are germline mutations of mismatch repair genes: *MLH1*, *MSH2*, *MSH6*, and *PMS2*. Mutations in *MLH1* and *MSH2* are the most frequent (about 90%) [4]. Compared to the general population, the lifetime risk of colon cancer in Lynch syndrome patients is 100-fold higher (1–4%) and the patients are on average 10 years younger [43, 44]. In addition, this genetic disorder also predisposes for other malignancies such as endometrial, ovary, genitourinary tract, stomach, hepatobiliary, pancreas, and small bowel cancers. Small bowel cancer can be the only cancer manifestation in Lynch syndrome patients [31]. Interestingly, the incidence of small bowel cancer in *MLH1* and *MSH2* carriers appears higher compared to *MSH6* [43]. Adenocarcinomas of the ampulla of Vater additionally belong to the spectrum of HNPCC [45].

Conclusion

Cancers of the ampullary/periampullary region represent a heterogeneous group of malignancies originating in a complex anatomical environment. A careful gross and histopathological assessment according to standardized protocols represents a first important step to differentiate these entities and their respective precursor lesions for appropriate classification and staging. This is mandatory considering the different biological behaviors, which further affect clinical management. In addition, exact morphological characterization represents the basis for further molecular analysis. Gene panel analysis might help in addition to conventional morphology and immunohistochemistry in the preoperative diagnosis of cancers of the ampullary/periampullary region, especially in case of cytological specimens or if the material is scarce. Moreover, recent data coming from ultra-deep sequencing studies have revealed on one side important correlations between mutational patterns and prognosis. On the other side, it seems that common genetic alterations, especially those involving the *WNT* pathway, can be found in subgroups of cancers of the

periampullary/ampullary region independently from the histotype and could be used for individual therapeutic approaches in the near future.

Key Research Points

- Neoplasms of the ampullary/periampullary region include ductal carcinomas of the pancreatic head, carcinoma of the ampulla of Vater, duodenal cancers, and distal bile duct cancers.
- Subtypes of cancers of the ampullary/periampullary region seem to originate from different precursors along different molecular pathways.
- A careful gross assessment of pancreatoduodenectomy specimens according to standardized protocols allows in most cases a differentiation between different cancer subtypes originating in the periampullary/ampullary region and is mandatory for determining prognostic relevant parameters, such as staging and resection margin status.
- Histopathological analysis accompanied by immunohistochemistry and molecular analysis is important for identification of special subtypes (e.g., intestinal vs. pancreatobiliary ampullary cancers, MSI vs. MSH neoplasms) with different prognosis and, possibly, different therapy response.

Future Scientific Directions

- Efforts are ongoing to elucidate further the molecular pathology underlying the observed histopathological and prognostic differences among cancers of the ampullary/periampullary regions.
- A better morphological and molecular characterization of the precursor lesions of different cancer types will also be helpful to better understand the biological behavior of cancers arising in the ampullary/periampullary region and their respective subtypes.
- Gene panel analysis should be exploited and possibly included in routine assessment in order to increase diagnostic sensitivity in the preoperative setting.
- NGS-based analyses have recently identified common molecular pathways in duodenal, ampullary, and distal bile duct cancers, which offer the possibility to apply individually targeted therapies.

Clinical Implications

- Despite similar approach for the treatment of resectable disease, the prognosis of cancers of the ampullary/periampullary region varies considerably depending on the tumor subtype, with survival rates being highest for ampullary and duodenal cancers, intermediate for distal bile duct cancer, and lowest for pancreatic cancer.

- Due to the differences in biological behavior and survival, accurate classification of carcinomas of the ampullary/periampullary region is crucial for an adequate prognostic estimate and an individualized therapeutic decision. Moreover, a subset of cancers of this region arises in a hereditary context (FAP or HNPCC), and these patients should therefore undergo a close surveillance program using upper gastrointestinal endoscopy.
- A better definition and characterization of precursor lesions, also on the molecular level, may improve early recognition, thereby significantly affecting treatment and prognosis.
- Molecular profiling should be exploited to increase diagnostic accuracy and to provide the basis for individual targeted therapies.

Cross-References

► Emerging Therapeutic Targets in Pancreatic Adenocarcinoma
► Precision Medicine Based on Next-Generation Sequencing and Master Controllers
► Smad4-TGF-β Signaling Pathways in Pancreatic Cancer Pathogenesis
► The Molecular Pathology of Precursor Lesions of Pancreatic Cancer

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Miscellaneous Nonpancreatic Nonendocrine Tumors

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Contents

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Abstract

Nonendocrine, nonpancreatic periampullary tumors are generally classified as arising from the ampulla of Vater, distal common bile duct, or duodenum. The most common clinical finding on presentation is obstructive jaundice. These lesions may occur spontaneously or as part of a hereditary syndrome (familial adenomatous polyposis, Gardner's syndrome, and inflammatory bowel disease). The most effective diagnostic strategies for determining extent of disease and resectability of periampullary tumors include dual-phase computed tomography and endoscopic ultrasound. Small, benign periampullary lesions may be amenable to endoscopic resection. For benign lesions <3 cm that are unable to be completely removed endoscopically, transduodenal local resection should be considered. Appropriate surgical candidates with larger lesions >3 cm or suspicion of invasive carcinoma should undergo a pancreaticoduodenectomy. Five-year survival for duodenal, ampullary, and distal common bile duct carcinomas are 51–59, 37–39, and 23–27%, respectively. For each of these tumors, both lymph node status and negative margins are significant predictors of outcome. At this point, neoadjuvant and adjuvant therapies have not clearly demonstrated a survival benefit for nonpancreatic periampullary cancers. The future success in treating these cancers likely rests in the development of novel biological and targeted therapies in the setting of well-designed multi-institutional clinical trials. This chapter will focus on benign and malignant nonpancreatic and nonneuroendocrine periampullary tumors and will include the pathology, clinical presentation, diagnostic workup, and management strategies to approach these neoplasms.

Keywords

Cholangiocarcinoma · Bile duct cancer · Ampullary adenoma · Ampullary cancer · Duodenal neoplams

Epidemiology

The majority of periampullary tumors are malignant, with pancreatic adenocarcinoma being the most common followed by cancers of the ampulla of Vater, distal common bile duct, and duodenum, respectively. Periampullary adenocarcinoma has a yearly incidence in the United States of approximately 35,000 cases which has remained stable over the last few decades [1]. Pancreatic adenocarcinoma likely accounts for up to 90% of these cases, although without surgical resection and pathologic analysis, the specific organ of origin can be difficult to determine. The relative frequency of malignant periampullary neoplasms in resected specimens is

Table 1 Relative frequency periampullary neoplasms in resected specimens

Location	Percentage (%)
Head of pancreas	56
Ampulla of Vater	21
Distal common bile duct	17
Duodenum	3

shown in Table 1 [2]. The percentage of nonpancreatic malignancies is likely higher in this surgical resection series given such tumors have a higher rate of resection compared to primary pancreatic cancers.

Pathology

Ampullary Neoplasms

Tumors of the ampulla can be either benign or malignant, both of which are rare. Autopsy studies demonstrate that the overall prevalence of ampullary adenomas is approximately 0.04–0.12% [3]. Among all malignancies of the gastrointestinal tract, ampullary neoplasms account for only 0.5% [4].

Benign Ampullary Tumors

Benign adenomas are generally defined as adenomatous lesions arising on or within 2 cm of the ampulla of Vater. They are classified by their microscopic findings as either intestinal type or biliary type. Ampullary adenomas are seen both sporadically and in association with familial syndromes such as familial adenomatous polyposis (FAP). The incidence of sporadic adenomas appears to be increasing, which is likely the direct result of increased detection due to the increased utilization of upper endoscopy. Sporadic ampullary adenomas usually occur during the sixth decade of life and are an average diameter of 2 cm [5, 6] (Fig. 1).

Ampullary adenomas are frequently identified in patients with FAP, who have a cumulative lifetime risk near 100% [3]. The median age at presentation for familial adenomas is earlier than the sporadic cases, presenting at 30–40 years of age. The diagnosis of periampullary adenomas associated with FAP usually occurs well after the diagnosis of colonic polyps typically at a mean follow-up of 17 years after colectomy [7]. At presentation, these lesions can often be multiple and involve both the ampulla and duodenal mucosal surface simultaneously.

Similar to the well-defined transformation of colonic adenomas into adenocarcinoma, ampullary adenomas have the potential for malignant degeneration. Patients with FAP have a 100–200-fold higher risk of periampullary cancer compared to the general population and a prevalence of ampullary cancer of 3–12% [4]. Thus, close screening and follow-up is extremely important in this population.

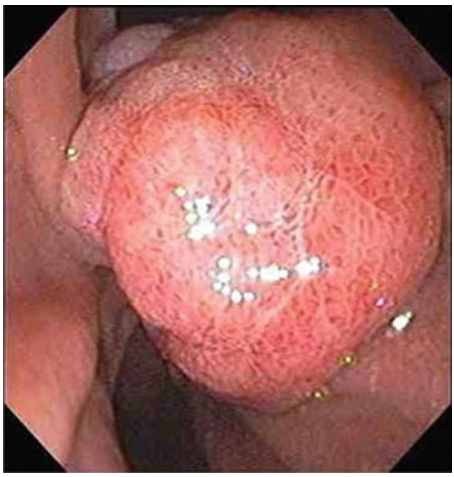

Fig. 1 Endoscopic appearance of benign villous adenoma

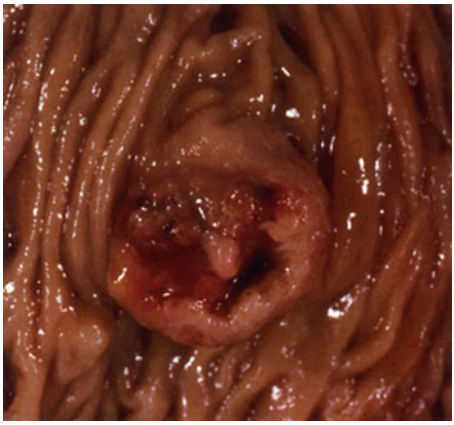

Fig. 2 Surgical specimen demonstrating an ulcerated ampullary carcinoma. The papilla is replaced by an exophytic papillary and ulcerated tumor (Reprinted from Mino and Lauwers [8])

Malignant Ampullary Tumors

Ampullary carcinoma is classified as four types based on macroscopic features: intra-ampullary (24%), periampullary duodenal (6%), mixed exophytic (31%), and mixed ulcerated (39%) (Fig. 2). Overall, intra-ampullary cancers have a better prognosis than other subtypes as these tumors usually present smaller lesions with less angiolymphatic invasion, fewer lymph node metastases, and less direct invasion of the pancreas.

Adenocarcinomas of the ampulla are further divided into intestinal-type (50%) and pancreaticobiliary type (20%) based on histologic features The most prevalent type of ampullary adenocarcinomas are intestinal-type which resemble primary adenocarcinomas of the colon pathologically with simple or cribriform glands lined by atypical cells with features of intraluminal necrosis and inflammation (Fig. 3). Pancreaticobiliary-type resembles primary pancreatic and biliary

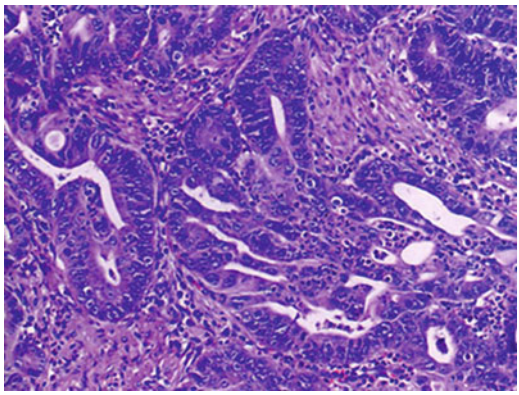

Fig. 3 Adenocarcinoma, intestinal type. The tumor is composed of complexed glands lined by atypical cells. Note the typical luminal inflammation (Reprinted from Mino and Lauwers [8])

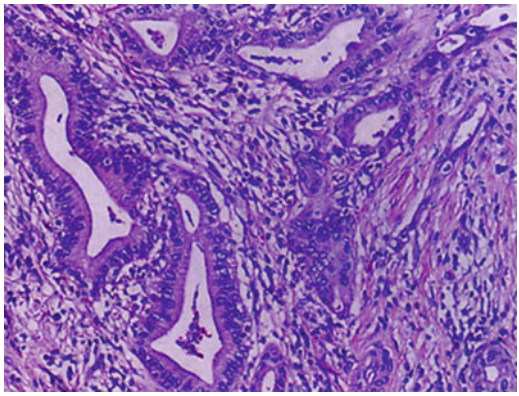

Fig. 4 Adenocarcinoma, pancreatobiliary type. The tumor is composed of simple malignant glands lined by low columnar cells. Note the markedly atypical nuclei and the surrounding desmoplasia (Reprinted from Mino and Lauwers [8])

adenocarcinomas. These tumors are composed of simple glands lined with low columnar cells with features of atypical nuclei and surrounding desmoplastic stroma (Fig. 4). Compared to the intestinal type, the pancreaticobiliary type more often demonstrates perineural invasion, but angiolymphatic invasion is less common. In instances where both microscopic features of intestinal and pancreaticobiliary are present, these tumors are classified as intestinal, unless there is a predominant pancreaticobiliary phenotype. A 2008 series from the University of California San Francisco of 118 patients with ampullary adenocarcinomas noted patients with pancreaticobiliary type presented with jaundice more frequently and had significantly worse survival compared to those with intestinal type [9].

There are several unusual subtypes of ampullary cancers including papillary, mucinous, and signet-ring carcinomas. Papillary carcinomas are uncommon and are reported in 6% of ampullary carcinomas. They are classified as either invasive or noninvasive. Invasive papillary carcinomas appear as complex branching papillary structures with fibrovascular cores and/or micropapillary structures without fibrovascular cores. These are lined by either intestinal or pancreatobiliary-type cells. In contrast, noninvasive papillary carcinomas are exophytic tumors arising in

the intra-ampullary mucosa and lined by pancreatobiliary-type epithelium. The neoplasms are similar to noninvasive papillary carcinomas of the extrahepatic bile ducts and noninvasive intraductal papillary mucinous neoplasms of the pancreas.

Mucinous or colloid carcinomas represent only 4–7% of ampullary carcinomas. These neoplasms demonstrate two particular morphologies both with greater than 50% containing extracellular mucin. These carcinomas are composed of columnar epithelium with nuclear atypia or contain clusters of neoplastic cells.

Signet-ring cell carcinomas of the ampulla are extremely rare. These neoplasms contain cells with nuclei forced to the periphery by intracytoplasmic mucin. In order to diagnose these tumors, greater than 50% of the tumors must contain signet-ring cells with a diffuse growth pattern, and a primary from another site must be excluded.

Distal Common Bile Duct Neoplasms

The common bile duct (CBD) is divided into four parts: (1) supraduodenal, (2) retroduodenal, (3) intrapancreatic, and (4) intraduodenal. The periampullary distal CBD is considered to include the intrapancreatic and intraduodenal segments. Tumors of epithelial, nonepithelial, and mesenchymal origin can arise from the distal CBD.

Benign Distal Bile Duct Tumors

Adenomas are extremely rare lesions of the distal common bile duct and are less common than carcinomas. These lesions are usually small, often single, and may appear as pedunculated or sessile polyps. They are histologically classified similarly to adenomas of the colon: tubular, tubulovillous, and villous. Reports of distal common bile duct adenomas have been reported in familial adenomatous polyposis and Gardner's syndrome [10].

Cystadenomas are mucinous cystic tumors that can arise from various structures in the upper gastrointestinal tract, most commonly seen in the liver, pancreas, and extrahepatic bile ducts. These tumors occur in the biliary tree of middle-aged females and may grow as large as 20 cm. Malignant transformation is rare, although dysplasia is seen in 13% of these tumors [11]. Complete local excision for symptomatic lesions is necessary due to a high rate of recurrence if incompletely resected. Cystadenomas can be differentiated from intraductal papillary mucinous neoplasms (IPMN) of the biliary tract by the presence of mesenchymal stroma in the former. Biliary IPMN, also seen originating from the pancreas, has emerged as a unique entity and may account for $>7\%$ of biliary neoplasms. These lesions are considered precursors to cholangiocarcinoma with risk of malignant transformation [12].

Biliary papillomatosis is a rare phenomenon of multicentric complex papillary neoplasms which involve the extra- and intrahepatic biliary systems and gallbladder, and may extend into the pancreatic ducts. It affects both males and females equally

during the sixth decade of life. Surgical resection is difficult and recurrence is common. The treatment of choice is total hepatectomy and liver transplantation.

Granular cell tumors are neoplasms of the extrahepatic biliary system usually involving the common bile duct. These tumors typically occur in young women (median age 34 years). Patients typically present with jaundice and abdominal pain. Granular cell tumors are occasionally multicentric with lesions in the gallbladder, skin, omentum, esophagus, and stomach. Within the common bile duct, these lesions appear as small (<2 cm), firm, submucosal nodules that invade the lumen. These tumors are not malignant, however may invade into periductal tissue and adjacent pancreas. Diagnosis usually occurs by ultrasound with subsequent MRCP/ERCP. These lesions are clinically similar to malignant distal common bile duct tumors and often require operative resection to make the diagnosis.

Malignant Distal Bile Duct Tumors

The incidence of extrahepatic cholangiocarcinoma in the United States is low, with approximately 6,000 new cases diagnosed annually [13]. Using the classification system proposed by Nakeeb and colleagues, cholangiocarcinoma lesions are divided into intrahepatic, perihilar, and distal subgroups [14]. Up to 45% of these malignancies are classified as extrahepatic [13]. Although the etiology is unknown, there are several well-documented risk factors. The incidence of all types of cholangiocarcinoma increases with age and is higher in males. Patients with ulcerative colitis and sclerosing cholangitis have a significantly increased risk of developing both intra- and extrahepatic cholangiocarcinoma at 65%, which is 4x higher than that of the general population. Although patients with Crohn's disease are at an increased risk for developing cholangiocarcinoma, the risk is approximately half of that for ulcerative colitis patients [15]. Biliary pathology such as cholangitis, choledocholithiasis, cholecystitis, and choledochal cysts are also independent risk factors for development of cholangiocarcinoma. Hepatolithiasis and biliary parasitic infestation (*Clonorchis sinensis* or *Opisthorchis viverrini*), both prevalent in parts of Asia, also increase the risk of cholangiocarcinoma.

Adenocarcinoma is the primary histologic subtype in the distal common bile duct malignancies. The three macroscopic classifications of cholangiocarcinoma are sclerosing, nodular, and papillary. Sclerosing lesions are the most common and appear as thickening of the bile duct with diffuse infiltration of adjacent tissues. Nodular tumors are irregular nodules that invade into the lumen of the bile duct. Nodular-sclerosing lesions, as implied, have characteristics of both. The papillary subtype represents only 10% of cholangiocarcinomas and is more common in the distal bile duct than the hepatic bifurcation [16]. These tumors are soft polypoid lesions with little or no invasive component and generally have a more favorable prognosis compared with the sclerosing subtype [17, 18]. These tumors spread longitudinally along the duct wall beneath the epithelial lining. As a result, preoperative imaging and intraoperative examination may not appreciate the extent of submucosal spread, highlighting the importance of intraoperative frozen section to determine adequate margins for resection (Table 2).

Table 2 Tumor classification table

Miscellaneous nonpancreatic nonneuroendocrine tumors	
Periampullary tumor classification	
1. Ampullary neoplasms	3. Duodenal neoplasms
A. Benign adenomas	A. Benign adenomas (tubular, villous, Brunner gland)
B. Adenocarcinomas (intestinal type, pancreaticobiliary type)	B. Lipomas
C. Papillary carcinoma (invasive, noninvasive)	C. Hamartomas
D. Mucinous or colloid carcinomas	D. Hemangiomas
E. Signet-ring carcinomas	E. Primary duodenal adenocarcinomas
2. Distal common bile duct neoplasms	4. Mesenchymal neoplasms
A. Benign adenomas	A. Leiomyomas, lipomas
B. Cystadenomas	B. Neurogenic tumors (neurofibromas, ganglioneuromas)
C. Biliary papillomatosis	C. Vascular tumors (hemangiomas, lymphangiomas)
D. Granular cell tumors	D. Granular cell tumors
E. Cholangiocarcinoma (sclerosing, nodular, papillary)	E. Schwann cell tumors
	F. Gastrointestinal stromal tumors (GIST)
	5. Lymphomas (B cell lymphomas)
	6. Metastatic tumors (renal cell carcinoma, melanoma, breast cancer, squamous cell carcinoma, endometrioid adenocarcinoma, osteosarcoma)
	7. Pseudotumors (myoepithelial hamartoma, Brunner gland hyperplasia)

Duodenal Neoplasms

Benign Duodenal Tumors

Small bowel tumors are rare and represent only 1–1.5% of all gastrointestinal neoplasms. Depending on the series, the proportion of benign small bowel tumors ranges from 14% to 52% [19]. Familial syndromes such as Gardner's syndrome and familial adenomatous polyposis are often associated with duodenal adenomas. Adenomas are comprised of three types: (1) tubular, (2) villous, and (3) Brunner gland. Tubular adenomas are usually pedunculated and generally have low risk for invasive carcinoma. Villous adenomas have a higher malignant potential, especially when greater than 2 cm. Brunner gland adenomas originate from hyperplastic exocrine glands in the proximal duodenum and carry no malignant risk.

Lipomas are rare tumors of the duodenum and are usually identified as incidental findings on CT as circumscribed tumors of fat density in the bowel wall. If symptomatic, they present as bleeding or obstruction. If small (<2 cm) and asymptomatic, they do not require resection. However, symptomatic, large or increasing size on serial CT requires endoscopic or segmental resection to rule out the possibility of liposarcoma.

Hamartomas are lesions seen almost exclusively in Peutz-Jeghers syndrome, an autosomal dominant condition characterized by multiple GI hamartomas throughout the bowel with mucocutaneous pigmentation. Rarely, these tumors cause obstruction or bleeding. Malignant transformation is rare but requires that these patients have close surveillance. Surgical intervention should be considered for symptomatic lesions or concern for the development of malignancy.

Hemangiomas are rare congenital lesions that present as acute or chronic bleeding during midlife. They are usually single and have no malignant potential. If these tumors are symptomatic, treatment consists of endoscopic or segmental resection. Additional treatment modalities including endoscopic sclerotherapy or angiographic embolization have also been described.

Malignant Duodenal Tumors

The incidence of small bowel cancer in the United States is approximately 10,000 cases per year with approximately 1300 deaths per year as a result [20]. The majority of small bowel adenocarcinomas arise in the duodenum and up to half of primary duodenal adenocarcinomas occur in the periampullary region [21, 22]. The incidence is higher in older patients and males more than females. Most cancers of the duodenum are sporadic. Familial adenomatous polyposis is the most prominent genetic predisposing factor with a relative risk of over 300 times that of the normal population. Hereditary nonpolyposis colorectal cancer, celiac sprue, and Crohn's disease are also associated with duodenal cancer.

Most of these tumors are solitary, sessile lesions, which often appear in association with adenomas. They are usually moderately well-differentiated. These lesions are similar to the malignant transformation of adenocarcinomas found in the colon with similar pathologic features.

Rare Periampullary Tumors

Mesenchymal Neoplasms

Benign and malignant periampullary mesenchymal tumors are extremely uncommon. The most common benign neoplasms are leiomyomas or lipomas. Other rare benign lesions consist of neurogenic tumors (neurofibromas, ganglioneuroma), vascular tumors (hemangiomas, lymphangioma), or granular cell tumors of Schwann cell origin. Neurogenic tumors involving the ampulla may arise in patients with neurofibromatosis.

Malignant mesenchymal tumors mostly consist of gastrointestinal stromal tumors (GISTs). Duodenal and periampullary stromal tumors compose about 3–5% of all GI stromal tumors [23]. The sub-proliferation in the majority of gastrointestinal stromal tumors is thought to be driven by gain-of-function mutations of the *KIT* gene, which encodes a type of tyrosine kinase receptor. Activating mutations of *KIT* can be found in most periampullary stromal tumors. These tumors can occur at any age and usually present with gastrointestinal bleeding associated with a growth of a large size with central necrosis. Complete surgical excision is the treatment of choice.

Because lymph node metastasis is rare, local resection can be employed selectively. Larger tumors, however, may require pancreaticoduodenectomy.

Lymphomas, Metastatic Tumors, and Pseudotumors

Other rare periampullary tumors include lymphomas and metastatic tumors. Most reports of lymphoma involving the ampulla of Vater involve high-grade B cell lymphoma and marginal zone B cell lymphoma. Metastatic disease involving the periampullary region is often from direct extension from an adjacent locally advanced tumor. Hematogenous spread from a primary neoplasm is extremely rare but most commonly reported with renal cell carcinoma. Other malignant tumors reported to metastasize to the periampullary region include melanoma, breast cancer, squamous cell carcinoma of the larynx, endometrioid adenocarcinoma, and osteosarcoma. Pseudotumors are recognized as 23% of tumors identified in the ampullary region [24]. They include myoepithelial hamartoma and Brunner gland hyperplasia, which collectively are more common than adenomas. It can be challenging to discern pseudotumors from neoplastic lesions, and often result to unnecessary surgery.

Clinical Presentation

Benign periampullary and duodenal adenomas are often asymptomatic and discovered incidentally or during surveillance for familial syndromes. Presenting symptoms depend on location and size of the tumors but can include jaundice, bleeding, and obstruction such as are seen with malignant periampullary tumors.

Generally, periampullary and pancreatic carcinomas are difficult to diagnose in their early stages. Symptoms tend to be nonspecific and often the diagnosis is not made until patients develop jaundice. Compared to pancreatic primaries however, tumors of the ampulla of Vater, distal common bile duct, and periampullary duodenum tend to present at an earlier stage due to higher propensity for biliary obstruction leading to jaundice. The mean diameter in one series of 149 patients diagnosed with ampullary cancer (2.7 cm) was significantly smaller, compared to pancreatic head cancer (3.5 cm) [25]. This generally translates to higher resectability rates than pancreatic cancers. Usually jaundice is progressive and relentless and may be associated with significant pruritus. Occasionally however, ampullary carcinomas may present with intermittent jaundice due to the "ball valve" effect of a polypoid tumor or necrosis during the growth phase leading to extrahepatic biliary obstruction. The development of jaundice is more commonly associated with a periampullary carcinoma (70%) than a benign tumor (20–30%) [26–30].

Periampullary neoplasms may also present with abdominal pain, anorexia, nausea, weight loss, and gastrointestinal bleeding. Partial biliary or pancreatic duct obstruction may result in complaints of abdominal pain prior to the development of jaundice. This pain is usually dull, moderate intensity, located in either the epigastrium or right upper quadrant, possibly radiating to the back, and aggravated by eating. Vomiting secondary to duodenal obstruction is usually a late manifestation of periampullary cancers in general, but may occur earlier in bulky duodenal cancers.

Ampullary or duodenal cancers may present with chronic or intermittent gastrointestinal bleeding. An episode of acute pancreatitis of unclear etiology should raise suspicion for an underlying periampullary neoplasm and initiate a thorough evaluation once the acute episode has resolved. In a report by Rattner et al., acute pancreatitis was the presenting symptom in 25% of patients diagnosed with ampullary neoplasms [31].

Duodenal adenocarcinomas not immediately adjacent to the ampulla of Vater may present with vague complaints of abdominal pain, weight loss, symptoms of bowel obstruction, or bleeding. These lesions tend to represent more advanced disease than periampullary adenocarcinomas.

Past medical and family history may be significant in evaluating a patient for a possible periampullary neoplasm. Patients with Gardner's syndrome and familial polyposis may carry a 200-fold increased risk for ampullary and duodenal carcinomas [32]. These patients will often have multiple polyps involving a significant portion of the duodenal mucosa.

Aside from jaundice, physical examination findings are commonly absent in patients with periampullary tumors. Hepatomegaly may be present and usually reflects hepatic congestion from biliary obstruction, not necessarily the presence of metastatic disease. Ascites, however, may represent advanced disease. A palpable gallbladder may be present in approximately 25% of patients. Occult fecal blood may be seen in those with bleeding periampullary cancers as well.

Diagnostic Evaluation

Laboratory Data

Nearly all patients with periampullary cancers present with abnormal liver function tests which includes increased plasma bilirubin and alkaline phosphatase, characteristic of extrahepatic obstruction. Transaminase levels may also be increased but usually not as significantly as alkaline phosphatase levels. In cases of longstanding extrahepatic obstruction, the prothrombin time may be prolonged. Anemia may be present with any periampullary cancers arising from the ampulla or duodenum secondary to gastrointestinal bleeding. Tumor markers, such as CEA and CA19-9, are generally not of diagnostic value as they are not specific for malignancy and may be elevated in benign causes of extrahepatic obstruction.

Imaging Studies

Early diagnosis of periampullary cancers is dependent on prompt evaluation of the jaundiced patient. Current imaging modalities provide detailed information regarding the level and etiology of biliary obstruction. Once these lesions are identified, a focused surgical approach gives these patients the best chance for long-term survival.

Ultrasonography

Transabdominal ultrasound (US) is often used in the initial evaluation of patients presenting with abdominal pain or obstructive jaundice, as it documents the presence of biliary obstruction with a dilated biliary tree and can define the level of biliary obstruction thereby narrowing the differential diagnosis. Other important findings that can be visualized with US include gallstones, ascites, and liver metastases. A major limitation of US is the frequent inability to identify a periampullary tumor and the high rates technically inadequate studies, which can result from patient body habitus, the presence of intervening bowel gas, or technical limitations of the operator. Conversely, the lack of radiation exposure and its relatively low cost are some of the advantages offered by US.

Computed Tomography

Despite the advantages of US, the high accuracy and reproducibility of computed tomography (CT) and its widespread availability, make it the most useful, and often the most cost-effective test in the initial evaluation of a patient with a suspected periampullary malignancy [33]. CT can detect the presence of a periampullary mass of at least 2 cm in size and also provides important information about the level of biliary obstruction with respect to the pancreatic parenchyma, if no mass is seen (Fig. 5). Pancreatic duct dilatation may also be seen. The optimal technique for evaluation of the periampullary region involves administration of both intravenous and oral contrast and obtaining 1- to 3-mm slices within a single breathhold during both the arterial and portal venous phase of intravenous contrast enhancement [34, 35]. Scans obtained during the rapid intravenous injection of an iodinated contrast agent result in an increase in the pancreatic parenchymal attenuation, as well as excellent contrast enhancement of the major peripancreatic blood vessels. This technique not only results in clear delineation of the tumor but may also demonstrate involvement of adjacent major visceral vessels, such as the portal/superior

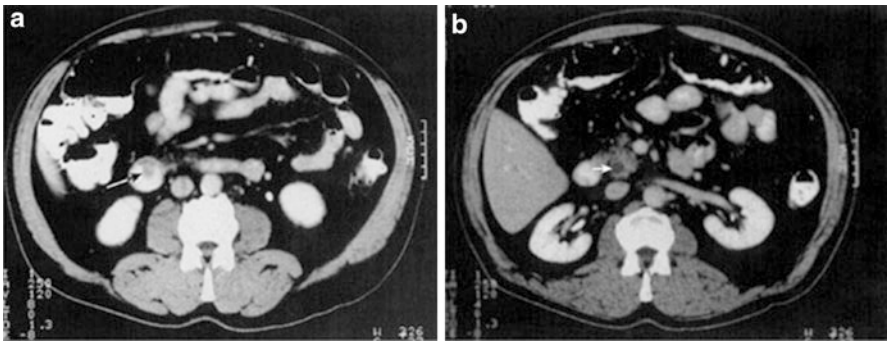

Fig. 5 Computed tomography scan of a patient with obstructive jaundice due to ampullary carcinoma: (**a**) Scan demonstrated a 3-cm ampullary mass (*black arrow*) and (**b**) scan at higher level demonstrating bile duct dilation within pancreatic parenchyma indicating distal duct obstruction (*white arrow*)

mesenteric vein complex or superior mesenteric or hepatic arteries, suggesting unresectability. The value of CT lies in the virtual absence of technically unsatisfactory examinations and in its high accuracy in both the detection and staging of periampullary carcinoma. The positive predictive value associated with CT-determination of unresectability is greater than 90% [34]. Magnetic resonance imaging (MRI) is equivalent to, but not superior to, CT for detection and staging of periampullary tumors and has a higher cost [36]. However, it does offer the advantages of avoiding exposure to radiation or ionic contrast and so is a more suitable test for patients with contrast allergies or renal insufficiency.

Magnetic Resonance Cholangiopancreatography

Magnetic resonance cholangiopancreatography (MRCP) has emerged as a noninvasive method to determine the most likely etiology of a pancreaticobiliary abnormality. It is most helpful in evaluating abnormalities of the proximal bile ducts and liver. In periampullary lesions, the thick slab MR images will delineate the biliary and pancreatic ductal anatomy with detail that is similar to the more invasive techniques of endoscopic retrograde cholangiopancreatography (ERCP). The other MR sequences will define the presence or absence of a mass, the level of the obstruction and the location of any given abnormality relative to the regional vessels.

The pattern on cholangiopancreatography can be characteristic for ampullary, bile duct, and pancreatic carcinomas. Cancers of the ampulla or duodenum will obstruct both the pancreatic and bile duct at the ampulla whereas pancreatic cancer will show the classic “double duct” sign. Distal bile duct cancers show a characteristic “apple core” appearance, with a normal appearing pancreatic duct.

Endoscopy/Endoscopic Ultrasound

Simple upper endoscopy can define the extent, size, and gross appearance of a periampullary lesion suspected of being malignant and allows for simultaneous performance of an endoscopic biopsy and cytologic brushings. The endoscopic appearance of an ampullary lesion, however, is often similar for benign and malignant tumors. Furthermore, endoscopic biopsies can reveal false negative results due to sampling error, with accuracy rates ranging from 62% to 79% in various series [37–40]. The demonstration of malignancy on biopsy specimens is definitive and will in most cases indicate the need for pancreaticoduodenectomy. However, a diagnosis of a benign adenoma does not rule out the presence of an adenocarcinoma elsewhere in the adenoma. Finally, an important consideration is that ampullary adenomas are a premalignant condition since they tend to progress to carcinoma. Therefore, regardless of whether the biopsy shows a malignant or benign histology, complete resection (either operative or endoscopic) is warranted.

Endoscopic ultrasonography (EUS) is a very useful modality in diagnosis of periampullary disease, which combines and modifies the techniques of gastrointestinal endoscopy and US. This combination decreases the distance between the ultrasonic source and the organ of interest, thereby markedly improving the resolution and imaging of the surrounding structures. Real-time EUS enables one to evaluate and integrate, on the same examination, mucosal, vascular, ductal, and

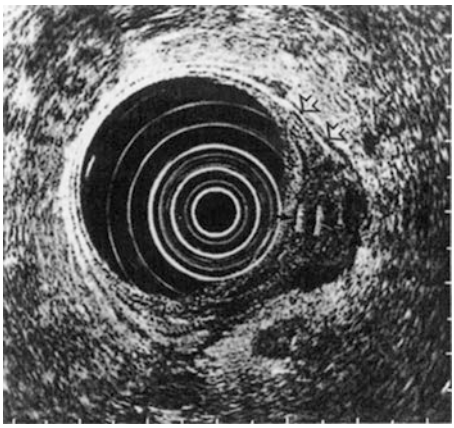

Fig. 6 Endoscopic ultrasonography scan of ampullary tumor, represented by the hypoechoic area on the *right*. An endoprosthesis (*small black arrows*) can be seen running through the center of the tumor. The tumor infiltrates beyond the muscularis propria (*open arrows*) into the pancreas.

parenchymal abnormalities. It allows detection of periampullary tumors, evaluation of their size and depth of invasion, as well as assessment of regional lymph nodes. EUS appears to be superior to CT and MRI for the detection of small pancreatic tumors (<2 cm) [41]. However, the sensitivity of EUS decreases in the setting of chronic pancreatitis [34]. EUS is able to demonstrate depth of invasion (T stage) of mucosal-based ampullary and duodenal tumors with an accuracy rate of 73–84%, increasing accuracy with higher T stages [42–44] (Fig. 6). This feature is of importance in detecting noninvasive benign periampullary neoplasms from malignant tumors with invasion through the bowel wall. Although results are not conclusive, several reports have also indicated that EUS has greater sensitivity and accuracy in detecting vascular invasion than CT [41, 43, 45].

The value of defining a benign versus a malignant periampullary mucosal-based tumor is the opportunity to locally excise a benign lesion as opposed to offer pancreaticoduodenectomy for malignant tumors. However, since frozen section analysis of resected specimens can fail to detect malignancy in 14% of patients [46], the surgeon always risks the possibility of a final diagnosis of cancer following local excision. Furthermore, with a sensitivity of approximately 75% in predicting T1 lesions, this modality is not necessarily optimal for predicting endoscopic resectability. Underestimating the depth of the tumor penetration seldom occurs, while overestimation is more common and is often due to edema of the submucosa from associated pancreatitis or from peritumoral inflammation in ampullary carcinoma. Similarly, indwelling transpapillary stents can cause inaccuracies and overstaging [47].

Finally, EUS can determine the presence or absence of enlarged regional lymph nodes. Reported accuracies of EUS-assessment of lymph node status have ranged from 63% to 84%, which is at least equivalent to CT [34, 41, 43, 48]. Furthermore, EUS offers the ability to perform fine-needle aspiration (FNA) of both the lesion and suspicious regional lymph nodes.

Limitations of EUS include its complexity and in operator variability in both performing and interpreting, its invasive nature, and its limited view (2–4 cm depth),

which does not allow evaluation for distant sites of metastases. The combination of CT and EUS is better than either alone in detecting resectability in patients with periampullary cancers. The strategy of obtaining a CT for all patients with suspected periampullary malignancies, followed by EUS in those patients in whom CT does not clearly demonstrate unresectability has been shown to be the most cost-effective strategy for preoperative staging of and determination of resectability in these tumors [45, 48].

Endoscopic Retrograde Cholangiopancreatography

With advances in cross-sectional imaging and the introduction of endoscopic ultrasound and MRCP, the role of endoscopic retrograde cholangiopancreatography (ERCP) in the diagnosis of periampullary lesions has become limited (Fig. 7). The

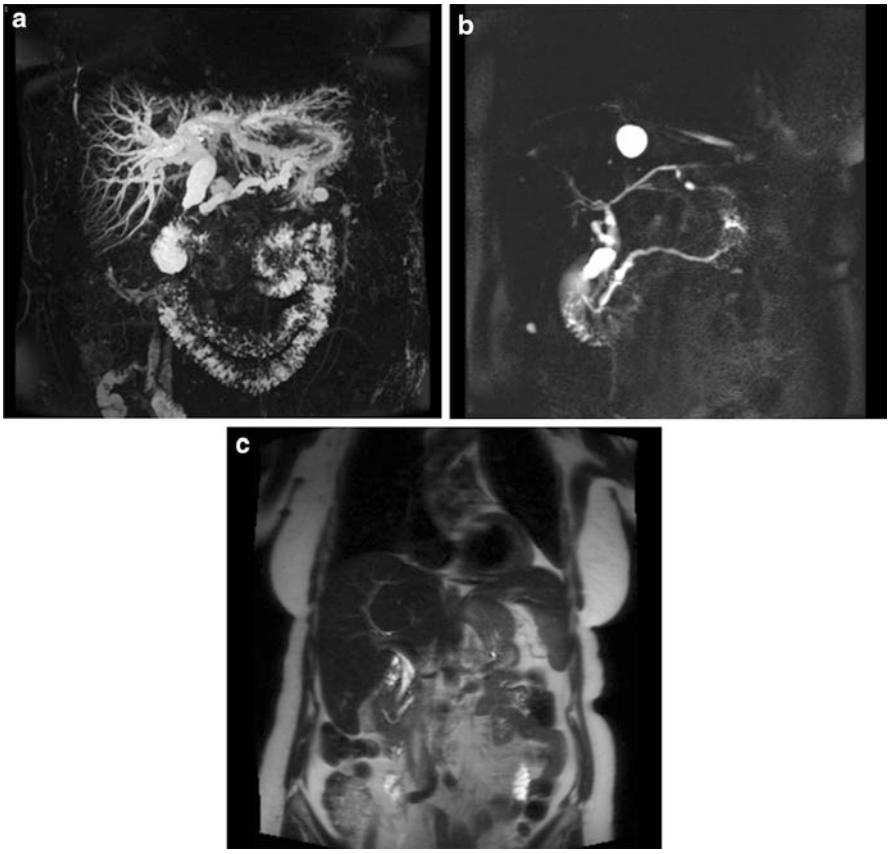

Fig. 7 (**a**) Magnetic resonance cholangiopancreaticogram (*MRCP*) showing an ampullary carcinoma obstructing the distal common bile duct, (**b**) MRCP with distal common bile duct carcinoma. Note the normal appearance of the main pancreatic duct, indicating a bile duct origin for the tumor, and (**c**) MRCP of a pancreatic carcinoma, with partial obstruction of both the main pancreatic duct and the common bile duct ("double-duct" sign)

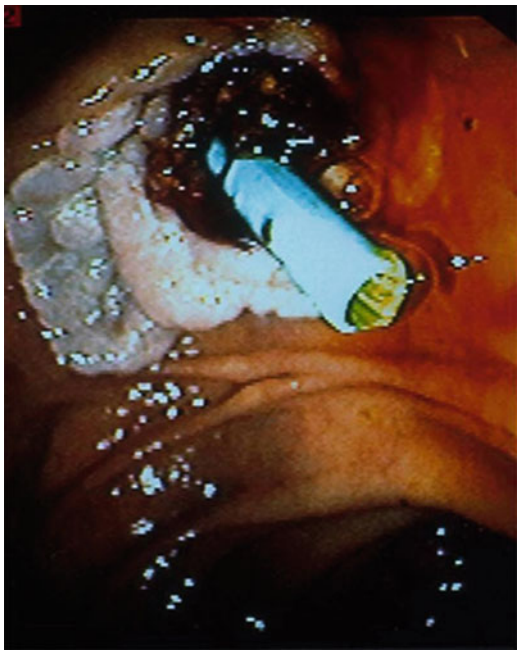

Fig. 8 Endoscopic photo of biliary stent placed through an obstructing ampullary carcinoma

most common current indication for ERCP in patients with periampullary tumors is for placement of a temporary stent in the common bile duct to relieve biliary obstruction preoperatively or as palliation (Fig. 8). Although stent placement will lead to colonization of the biliary tree and a higher perioperative infection rate in resected patients, it is appropriate in a number of clinical circumstances: (1) patients who present with symptoms of cholangitis requiring immediate intervention to treat the biliary infection, (2) patients presenting with intractable pruritus that can be relieved during the period of preoperative evaluation, and (3) patients with hyperbilirubinemia associated with renal insufficiency, which will correct with relief of the biliary obstruction. Under these circumstances, at least 2–3 weeks should be allowed prior to definitive resection to allow the metabolic derangements to normalize and to ensure the absence of active infection after instrumentation. Endoscopic stenting can provide relief of jaundice in patients in whom delay in surgery may be necessary to allow referral to a high-volume institution or for planned neoadjuvant therapy.

A final role for ERCP is to provide a tissue diagnosis of malignancy by either histology or cytology through direct brushings. A tissue diagnosis is valuable primarily for isolated bile duct strictures in clinical settings in which a benign etiology of the stricture would alter management (resection vs. stenting or bypass). In selected cases, Spyglass technology, essentially a smaller scope inserted the working channel of a standard ERCP scope, can allow directed visualization of the stricture for biopsy [49].

Percutaneous Transhepatic Cholangiography

As with ERCP, percutaneous transhepatic cholangiography (PTC) for diagnosis of a periampullary malignancy is seldom indicated due to the availability of noninvasive techniques. It does remain a therapeutic option in some patients, particularly when the endoscopic route is unsuccessful due to complete obstruction of the ampulla by tumor or the ampulla is not accessible due to a prior surgical procedure such as gastric bypass. PTC is often technically easier with a dilated biliary tree and is useful in defining the proximal biliary system, which is critical in the decision-making process concerning biliary reconstruction. Percutaneously placed catheters can be helpful during operative management for either resection or palliation, especially in reoperative cases or in the early postoperative period to allow biliary decompression in order to protect the biliary anastomosis. In most patients, however, PTC offers little advantage over ERCP, has a greater morbidity, and should be considered only if ERCP is technically not possible.

Preoperative Staging

The goal of preoperative staging is to determine which tumors are potentially resectable and have not already metastasized to distant sites or directly invaded the major peripancreatic vessels. This is more important in patients with pancreatic periampullary neoplasms because of the lower rate of resectability in this group. In the past, laparotomy was required in all patients to establish the diagnosis and, thereafter, resection or operative palliation was performed. Today, modalities including dual-phase CT, EUS, and diagnostic laparoscopy allow us to clinically stage patients preoperatively. The dynamic spiral CT scan is currently the most valuable of these studies, playing a role in both diagnosis and staging of periampullary neoplasms. Its primary advantages are the lower cost and noninvasive nature of the technique. Computed tomography can detect liver metastases (>1.0 cm) or larger peritoneal implants [34]. EUS has high accuracy for evaluating T stage and defining malignancy by demonstrating invasion. The technique can also be used to perform an FNA for histologic evaluation of suspicious lymph nodes. However, EUS cannot be used as the sole modality for staging. Given its inability to adequately rule out peritoneal or hepatic metastases, it should be combined with CT or laparoscopy for complete staging.

One of the limitations of CT is its poor sensitivity for detecting lesions in the liver, omentum, or peritoneal surface that are less than 1 cm in size. In an attempt to identify such metastases in a minimally invasive manner, laparoscopy has been suggested as a method for further staging. A recently published Cochrane review of 16 studies and a total of 1,146 patients with pancreatic or periampullary cancer suggests that the addition of diagnostic laparoscopy decreases the rate of unnecessary laparotomy in those patients deemed resectable on CT scan by 20% [50]. However, this review includes studies with dates ranging from 1986 to 2014, and CT has become more effective at picking up suspicious small volume metastases with

dual-phase imaging. Furthermore, the yield of diagnostic laparoscopy is likely lower for patients with ampullary and duodenal tumors than those with pancreatic cancer, leading many surgeon to avoid this step in patients with these tumors [51, 52].

The decision to stage patients with periampullary neoplasms via laparoscopy is largely dependent on the treatment algorithms of the surgeon. Those surgeons favoring surgical palliation as opposed to nonoperative palliation of unresectable tumors consider laparoscopy unnecessary. Whereas those surgeons who feel endoscopic palliation is adequate for most patients suggest that laparoscopy can save a substantial number of patients from the morbidity of a noncurative laparotomy. Those centers currently investigating neoadjuvant chemotherapeutic and radiation protocols also feel that laparoscopy is important in order to document the absence of liver or peritoneal metastases. Improvements in preoperative imaging and the addition of EUS to our clinical armamentarium has allowed for better selection of patients for operation with fewer patients being found to be unresectable at the time of operation, thereby minimizing unnecessary morbidity. Nonoperative techniques for the management of obstructive jaundice secondary to a periampullary tumor have also improved and can provide adequate palliation for most patients with unresectable neoplasms. However, as mentioned, patients with nonpancreatic periampullary neoplasms typically present earlier in the progression of their disease and have a much higher rate of resectability, thus in many cases preoperative staging with the currently available imaging modalities is sufficient.

Surgical Management

Endoscopic Resection

Benign periampullary tumors and small, ampullary tubular adenomas with very low malignant potential may be endoscopically resected. Small, pedunculated adenomas of the distal common bile duct can also be successfully treated and excised endoscopically. For tubular duodenal and Brunner gland adenomas, endoscopic excision is the most suitable option. With villous duodenal adenomas, transduodenal local excision should be considered depending on the size of the lesion. Endoscopic resection of a villous adenoma may be performed only if the entire lesion can be safely removed (Fig. 9). Close follow-up with repeat endoscopy is indicated in such cases, as recurrence rates can be seen in 10–25% of cases [53]. Finally, it is reasonable to consider endoscopic resection as a palliative option with patients that cannot tolerate general anesthesia to perform even a local excision for periampullary cancers.

Complications following endoscopic resection of ampullary tumors includes pancreatitis (5–15%), bleeding (4–15%), perforation (<2%), and cholangitis (<2%). Mortality, however, remains very uncommon.

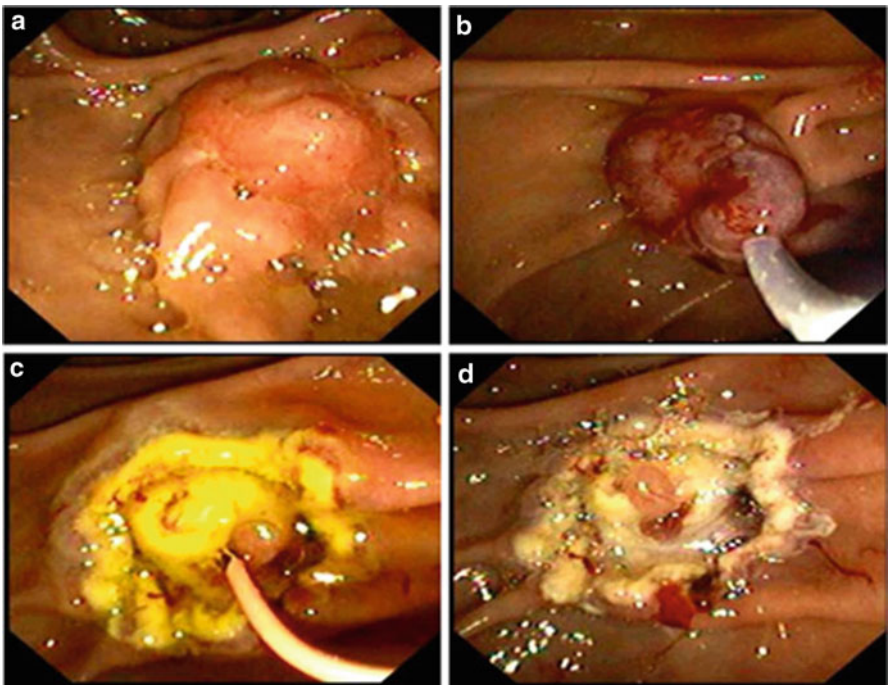

Fig. 9 (**a**) Endoscopic appearance of a benign periampullary adenoma, (**b** and **c**) endoscopic cautery excision of lesion, and (**d**) final appearance after complete endoscopic excision

Local Excision

Local resection of an ampullary tumor with reimplantation of the pancreatic and common bile ducts was first described by Halsted in 1899. Initially, this procedure was associated with high operative mortality and low long-term survival; however, with improvements in technique and preoperative staging, transduodenal ampullary resection has regained popularity. Local resection of the ampulla of Vater has been suggested for benign ampullary tumors or low grade ampullary carcinomas. Histologic confirmation of malignancy, large size, or extension into the common bile duct or pancreatic duct precludes local excision. Furthermore, the false negative rate of endoscopic biopsy (up to 25%) or even intraoperative frozen section (up to 14%) requires that complete histologic diagnosis of the entire resected specimen be completed [37, 46]. If invasive cancer is found in permanent sections, subsequent resection with pancreaticoduodenectomy is necessary.

The operation begins with an exploration of the abdomen through a right subcostal or upper midline incision to rule out metastatic disease. An extended Kocher maneuver is performed to mobilize the duodenum. A longitudinal duodenotomy is made over the junction of the second and third portions of the duodenum. Stay sutures are placed to expose the ampullary lesion, and the common bile duct is

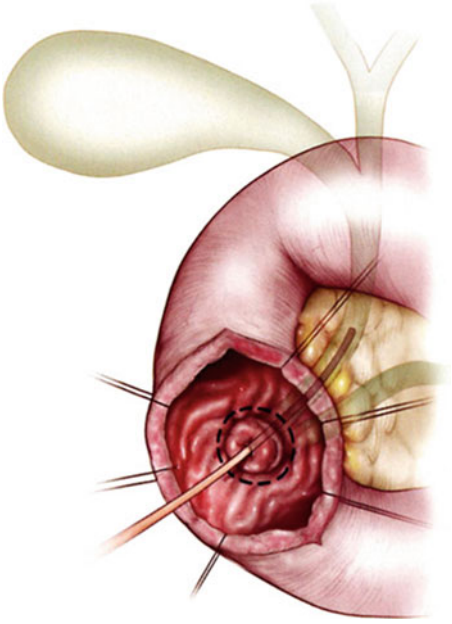

Fig. 10 The ampulla is exposed via a longitudinal duodenotomy, and the common bile duct is cannulated (Reprinted from Clary et al. [54])

cannulated through the center of the mass. If the common bile duct cannot be directly entered, passage of a biliary Fogarty catheter from above via cannulation through the cystic duct following cholecystectomy is advisable. Next, a resection margin of 0.5–1.0 cm of normal tissue is created by scoring the mucosal surface with electrocautery (Fig. 10). The lesion is excised by dissecting lateral to medial in the submucosal plane. In this approach, the common bile duct located at 11 o' clock, is transected prior to the pancreatic duct and located at 5 o' clock. The specimen is sent to pathology for frozen-section analysis. If a negative margin is not accomplished or an invasive component is identified, then a pancreaticoduodenectomy should be performed. In a series of 39 patients undergoing ampullectomy at Duke University Medical Center, the negative predictive values of frozen-section analysis was 94% [29]. If the lesion is benign and negative margins are achieved, then the common channel between the common bile duct and pancreatic duct is reconstructed by dividing the intervening septum with scissors. Next, the circumferential anastomosis between the duodenal mucosa to the common channel is performed with 5-0 Vicryl interrupted sutures. Lastly, the duodenum is closed transversely in two layers.

Recurrence rates after local excision in patients with sporadic adenomas are 0–26% [28–30, 54, 55]. Significantly, increased rates of recurrences are seen in patients with polyposis syndromes and approximately 25% of all recurrences are invasive carcinomas [30]. This highlights the importance of surveillance endoscopy following ampullectomy. Most series demonstrated complication rates of 20–25% which included delayed gastric emptying, duodenal leak, pancreatitis, cholangitis, and common bile duct stricture.

Pancreaticoduodenectomy

Since its introduction by Whipple et al. in 1935, pancreaticoduodenectomy has been the most effective treatment for periampullary carcinomas [56]. Either classic or pylorus-preserving pancreaticodenectomy is appropriate for most periampullary cancers, with the exception of patients with extensive duodenal polyposis associated with FAP. In such cases all duodenal mucosa should be removed, and therefore the total duodenectomy approach of the classic resection is appropriate. A prospective randomized study by Yeo and colleagues, showed no advantage to an extended retroperitoneal lymphadenectomy when performing a pancreaticoduodenectomy for periampullary adenocarcinomas including ampullary and distal common bile duct primaries [57].

Perioperative morbidity and mortality rates have continued to improve over the past decade with mortality rates of 2% or less and morbidity rates of 30–40% expected in patients treated at high volume centers [58–60]. One of the complications of pancreaticoduodenectomy that may be slightly increased in nonpancreatic tumors is the rate of pancreatic anastomotic leak due to the normal, soft texture of the pancreas. On the other hand, since local vascular invasion by periampullary nonpancreatic tumors is uncommon, the procedures are often technically easier.

To date, no study has directly compared local ampullary resection with pancreaticoduodenectomy for small ampullary cancers. There are several series including subsets of patients with T1 lesions for whom local resection was performed, usually in high-risk patients that were poor candidates for the more radical resection [28, 31, 61, 62]. Although these subsets are not prospectively randomized, patients that underwent pancreaticoduodenectomy for T1 tumors generally experienced both higher disease-free and overall survival rates [27, 28, 63, 64]. As a result, local excision is only acceptable for patients with small ampullary cancers that are unable to tolerate a pancreaticoduodenectomy. Refer to the clinical algorithm in Figure 11 for the evaluation and management of periampullary tumors (Fig. 11).

Segmental Resection

Surgery options for duodenal adenocarcinomas include segmental resection and pancreaticoduodenectomy. For lesions involving the proximal first and second portions of the duodenum, the treatment of choice is a Whipple procedure. Patients with more distal tumors involving the third and fourth portions of the duodenum, an en bloc segmental resection of the distal duodenum and proximal jejunum with lymphadenectomy is appropriate. Past studies have demonstrated that pancreaticoduodenectomy has an improved disease free interval and overall survival compared to segmental resections. This difference was most likely due to the earlier detection of periampullary duodenal adenocarcinomas than more distal tumors. More recent research suggests that although radical resection with pancreatiocduodenectomy is associated with a greater number of lymph nodes sampled, the overall survival is the same as with segmental resection [65, 66].

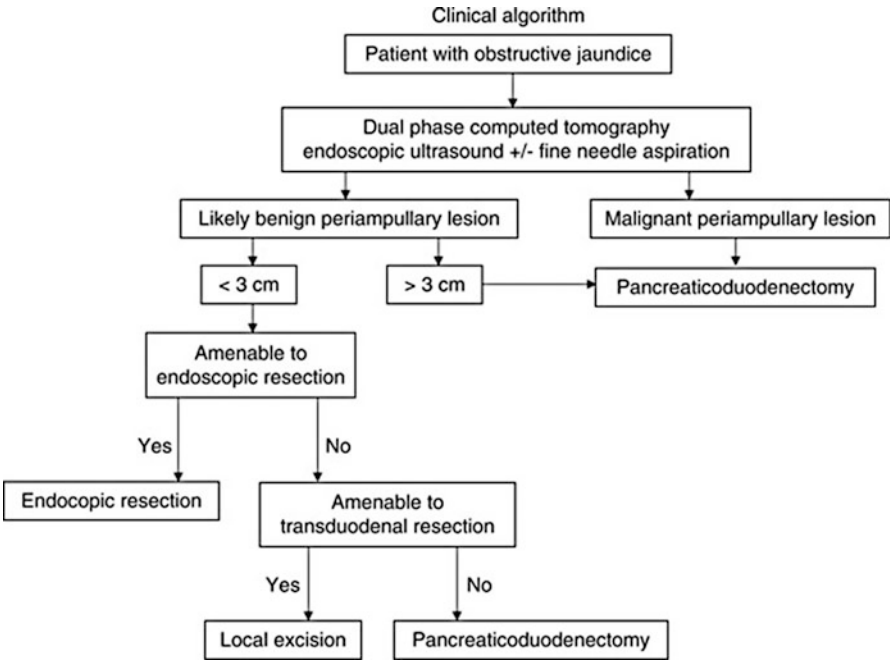

Fig. 11 Clinical algorithm for the evaluation and management of a periampullary lesion. Note: If invasive carcinoma discovered on endoscopic or local resection, proceed with pancreaticoduodenectomy

Palliative Procedures

In patients with unresectable or metastatic disease found at exploration, palliative operative gastric or biliary bypass should be strongly considered and performed especially if patient is symptomatic. For those with recurrent disease or known metastatic disease prior to exploration, palliative biliary stents and duodenal wall stents placed endoscopically may be the most appropriate local therapy to relieve symptoms and avoid delaying any additional systemic therapies being considered. In patients with bulky bleeding tumors, gastrojejunostomy (potentially performed laparoscopically), and radiation therapy can usually control symptoms.

Adjuvant and Neoadjuvant Therapy

The use of adjuvant and neoadjuvant therapies for nonpancreatic periampullary cancers has been reported. Due to the rarity of these lesions, most series remain low-powered and nonrandomized. Nevertheless, the use of neoadjuvant strategies for treatment of periampullary malignancies is becoming more popular. These approaches are mostly observed with pancreatic adenocarcinoma as very little

published data exists at this point regarding nonpancreatic periampullary primaries. The theoretical advantages include the delivery of a systemic therapy to well-oxygenated tissues, and the potential for down-staging unresectable and borderline resectable lesions. In multiple series, neoadjuvant chemoradiation did not increase the mortality or morbidity of pancreaticoduodenectomy for periampullary cancers, and interestingly yielded fewer pancreatic leaks and leak-associated morbidity and mortality compared to those not receiving neoadjuvant therapy [67, 68]. Critics of neoadjuvant protocols for potentially resectable periampullary cancers point to selection biases based on favorable biology in those that proceed on to resection following chemoradiation treatment.

The role of adjuvant therapy in ampullary cancer has been assessed in numerous small studies. In a series from Stanford, 12 patients with resected ampullary cancers having lymph node metastases, positive margins, tumor size >2 cm, poorly differentiated, or neurovascular invasion were given adjuvant chemoradiation resulting in an 89% actuarial 1-year survival [69]. In another series from Johns Hopkins, 17 of 106 patients with a resected ampullary cancer received adjuvant therapies without any survival benefit [26]. In the European Organization for Research and Treatment of Cancer (EORTC) Trial 40,891, there was no benefit of adjuvant chemoradiation over observation for nonpancreatic periampullary malignancies [70]. Finally, a recent meta-analysis of ten retrospective studies, including 3,361 patients, has demonstrated adjuvant chemoradiation therapy improved overall survival [71]. Due to these mixed results, chemotherapy with regimens similar to those used for colon cancer rather than the more aggressive chemoradiation protocols.

Adjuvant therapies for cholangiocarcinoma are also not well defined. A Japanese randomized, multi-institutional trial of 139 patients with bile duct cancer showed no difference in 5-year survival for patients receiving adjuvant chemotherapy [72]. In contrast, there is some data to support its use from a recent retrospective study. A Johns Hopkins study from 1994 to 2003, treated 34 patients with distal bile duct adenocarcinomas with pancreaticoduodenectomy followed by adjuvant chemoradiation and compared with historical controls from the same institution. For both lymph node positive and negative patients, overall survival was improved in patients that received surgery plus adjuvant chemoradiation [73]. There are a few prospective, randomized trials ongoing to determine the role of adjuvant chemoradiation for biliary tract cancer [74].

Due to the relatively rare incidence of primary duodenal adenocarcinomas, current data regarding its utility has not been able to identify a role for adjuvant therapy. The group at Johns Hopkins published a small retrospective series of 14 patients with stage III/IV periampullary adenocarcinoma of the duodenum that were treated with pancreaticoduodenectomy and adjuvant chemoradiotherapy. Comparing their results with historic controls, there was no difference in overall 5-year survival between surgery plus adjuvant chemoradiation versus surgery alone [75]. Despite the lack of data to justify adjuvant therapies for primary duodenal adenocarcinoma at this time, most medical oncologists would recommend its use for advanced stage disease.

Finally, the European Study Group for Pancreatic Cancer (ESPAC) – 3 trial was reported in 2012 [76]. This open-label phase 3 randomized controlled trial involving 100 centers included 428 patients with resected nonpancreatic periampullary cancers and compared adjuvant therapy via multiple regimens with observation. The results were mixed with adjuvant chemotherapy not showing significant survival benefit over observation (43.1 months vs. 35.2 months). However, multivariable analysis adjusted for prognostic variables did show significant survival benefit with adjuvant chemotherapy.

Survival

Overall, the survival following surgical resection for nonpancreatic periampullary cancers are substantially better than periampullary pancreatic cancer (Fig. 12). In the series from Johns Hopkins, duodenal and ampullary cancers demonstrate the 5-year survival rates of 51–59 and 37–39%, respectively [2, 77]. In the same series, distal cholangiocarcinomas and pancreatic cancers have the lowest 5-year survival rates, at 23–27 and 15–17%, respectively. In the Memorial Sloan Kettering experience, ampullary carcinomas had the highest overall survival rates (median 43.6 months) and resectability (82.1%) for periampullary tumors [64]. Beger et al. reviewed 171 cases of consecutive ampullary cancer treated by local or radical resection. The 5-year survival rates by stage in that series were 84% (stage I), 70% (stage II), and 27% (stage III) and 0% (stage IV) [28]. Poor prognostic indicators for recurrence

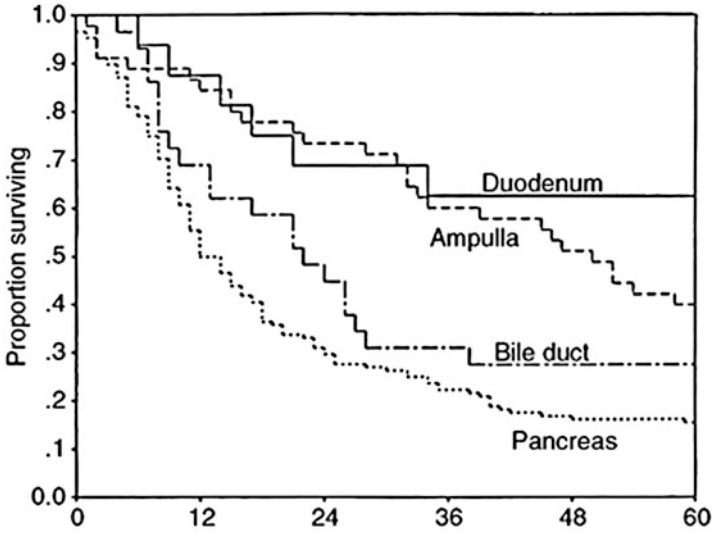

Fig. 12 The tumor-specific actual 5-year survival curves for the cohort of 242 patients treated by pancreaticoduodenectomy for periampullary adenocarcinoma (Reprinted from [77])

after resection of ampullary adenocarcinoma are advanced T stage, lymph node involvement, positive margins, neural invasion, and poor differentiation [26, 27, 63, 64]. The two most important factors commonly found among different series are T stage and nodal status, where the rate of lymph node involvement is a reflection of the T stage progression. For T1–T2 and T3–T4 tumors the percent of lymph node positivity is approximately 20 and 50%, respectively [28]. However, as previously stated, there is no advantage to an extended retroperitoneal lymphadenectomy when performing a pancreaticoduodenectomy for any periampullary adenocarcinoma [57].

With complete resection of distal cholangiocarcinoma, 5-year survivals range from 21% to 54% [78–80]. Resection rates are generally between 40% and 85%. In the series from DeOliveira et al., reviewing 564 patients with bile duct cancer undergoing surgery, the 239 patients with distal cholangiocarcinoma had an overall 5-year survival for all patients and those after R0 resection were 23 and 27%, respectively. The significant predictors of survival for patients with distal cholangiocarcinoma included negative margins, lymph node involvement, size >2 cm, and degree of differentiation [81]. The Japanese have compiled their extensive experience of distal cholangiocarcinomas into a national registry demonstrating a similar 5-year survival of 26% [82].

The most significant predictors of long-term survival for primary duodenal carcinoma include margin negative resection and lymph node involvement. For node-negative patients, overall 5-year survival following resection varies from 38% to 83%. For node-positive patients, the 5-year survival drops to 15–56%. In a 2000 series from the Mayo Clinic of 101 consecutive patients undergoing surgery for adenocarcinoma of the duodenum, lymph node involvement, stage III or greater, positive margin, and weight loss each carried a significantly negative impact on survival [83]. In the same series, the tumor grade, size, and location within the duodenum had no impact on survival (5-year survival, 54%). In the Memorial series, the survival benefit between node-positive (5-year survival, 56%) and node-negative (5-year survival, 83%) tumors demonstrated in patients with ≥ 15 nodes sampled did not carry a similar positive prognostic impact on survival when <15 lymph nodes were sampled [84]. The Hopkins group published their retrospective experience of 55 patients surgically treated primary adenocarcinoma of the duodenum [85]. Similar to other series, the 5-year survival was 53%. In this series, negative margins, pancreaticoduodenectomy, and tumors involving the first and second portions of the duodenum were favorable predictors of long-term survival. Nodal status, tumor diameter and grade did not influence survival in this study.

Conclusion

Nonendocrine, nonpancreatic periampullary tumors are rare lesions that encompass a large array of pathology, originating most commonly from the ampulla of Vater, distal common bile duct, and duodenum. These tumors are often asymptomatic and have a tendency to be malignant. Treatment options depend on size and malignant potential, ranging from endoscopic resection to pancreaticoduodenectomy. Further

research is required both to better understand the molecular biology of periampullary tumors and the role of perioperative chemotherapy.

Key Practice Points

- Nonpancreatic periampullary malignancies originate from the distal common bile duct, ampulla of Vater, and duodenum.
- Clinical findings of biliary obstruction require cross-sectional imaging and evaluation of the biliary system to exclude periampullary malignancies.
- Patients with familial syndromes (FAP, Gardner's syndrome, inflammatory bowel disease) must undergo close surveillance for periampullary cancers.
- Preoperative staging with CT and endoscopic ultrasound are the most cost-effective diagnostic strategies for determining resectability.
- In general, due to the higher rate of resectability of nonpancreatic periampullary neoplasms, preoperative staging and laparoscopic exploration are less important than pancreatic primary tumors.
- Small, benign periampullary lesions may undergo endoscopic resection.
- Transduodenal resection should be considered for small (<3 cm) ampullary tumors or low grade ampullary malignancies in patients unable to tolerate a pancreaticoduodenectomy.
- For large periampullary lesions (>3 cm) and invasive periampullary malignancies, pancreaticoduodenectomy remains the standard treatment.
- Adjuvant and neoadjuvant treatments for nonpancreatic periampullary tumors have been investigated; however, no clear survival benefit has been identified.
- Actual 5-year survival rates following surgical resection for nonpancreatic periampullary cancers as 51% for duodenal cancer, 37% for ampullary cancer, and 23% for distal common bile duct cancer.
- Poor prognostic indicators for ampullary adenocarcinoma include advanced T stage, lymph node involvement, positive margins, neural invasion, and poor differentiation.
- Significant predictors of survival for distal cholangiocarcinoma include negative margins, lymph node involvement, size >2 cm, and degree of differentiation.
- Significant predictors of survival for duodenal carcinoma include margin negative resection and lymph node involvement.

Future Research Directions

- Translational investigations to better understand the molecular biology of periampullary tumors to improve early detection and targeted therapies.
- Technologic advances to improve local endoscopic diagnosis, staging, and management.
- Multi-institutional clinical trials to investigate adjuvant and neoadjuvant therapies for resectable periampullary cancers.

Cross-References

- ▶ Chemotherapy for Advanced Pancreatic Cancer
- ▶ Clinical Decision-Making in Pancreatic Cancer
- ▶ EUS and Its Role in Pancreatic Cancer
- ▶ Laparoscopic Surgery for Pancreatic Neoplasms
- ▶ MRI and MRCP for Diagnosis and Staging of Pancreatic Cancer
- ▶ Surgical Resection for Pancreatic Cancer Using the International Study Group of Pancreatic Surgery (ISGPS) Classifications
- ▶ Therapeutic Endoscopy in the Management of Pancreatic Cancer

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Animal Modeling of Pancreatitis-to-Cancer Progression

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Abstract

Inflammatory diseases are the most common conditions of the exocrine pancreas. Chronic pancreatitis is often the result of recurrent bouts of acute pancreatitis and is a risk factor for pancreatic cancer. There has been a long interest in modeling the pathophysiological relationship between chronic pancreatitis and cancer and the recent development of genetic mouse models of pancreatic diseases has accelerated the discovery of mechanistic insights. The current paradigm proposes that the inability of normal pancreatic cells to recover from injury establishes a biological landscape that promotes cancer development. Multiple types of mechanisms concur in this process, in which both epithelial and nonepithelial cells participate, leading to persistent inability of epithelial cells to restore their differentiation programs. Developmental pathways involved in pancreatic differentiation are subverted to maintain cellular phenotypes that promote signaling from mutant KRAS, preneoplasia, and neoplasia. Downstream from KRAS, and in parallel with it, tyrosine kinase receptors, the MAPK, PI3K, NF-KB, and STAT pathways, and the mechanisms that control senescence and autophagy, contribute to the emergence of transformed clones. These signaling pathways, whose activity is modulated through complex cross-talks between epithelial, mesenchymal, and inflammatory cells, play crucial roles in the pancreatitis-to-cancer progression and provide opportunities for intervention in high-risk patients.

Keywords

Pancreatitis · Pancreatic cancer · Caerulein · Acino-ductal metaplasia

List of Abbreviations

ADM	Acinar-to-ductal metaplasia
AP	Acute pancreatitis
CCK	Cholecystokinin
CCKR	CCK receptor
CDE	Choline-deficient, ethionine-supplemented diet
CFTR	Cystic fibrosis transmembrane conductance regulator
CP	Chronic pancreatitis
ECM	Extracellular matrix
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
EUS-FNA	Endoscopic ultrasound-guided fine needle aspiration
GEMM	Genetically engineered mouse models
IPMN	Intraductal papillary mucinous neoplasm
JAK	Janus-activated kinase
LPS	Lipopolysaccharide
PanIN	Pancreatic intraepithelial neoplasia
PDAC	Pancreatic ductal adenocarcinoma
PDL	Pancreatic duct ligation
PSC	Pancreatic stellate cells

TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
WT	Wild type

Introduction

Acute and chronic pancreatitis are the main inflammatory disease of the pancreas. Current thoughts support the existence of a continuum whereby a relatively low proportion of patients who develop a first episode of acute pancreatitis go on to develop recurrent bouts of the disease that eventually lead to persistent unrepaired damage. Chronic pancreatitis (CP) has been recently defined as "a progressive inflammatory disease, which leads to loss of pancreatic function and other disease-associated morbidities" [1]. Chronic pancreatitis is a well-established risk factor for the development of pancreatic ductal adenocarcinoma (PDAC) [2], providing a strong argument supporting the role of damage/regeneration cycle and inflammation in tumor development. Recent evidence also supports that it is a risk factor for intraductal papillary mucinous neoplasms (IPMNs) [3]. The increase in risk is much higher for patients with hereditary chronic pancreatitis, caused by mutations in genes involved in the regulation of trypsin activation (up to 70-fold) [4]. However, such cases are very rare. The fold-increase risk of developing PDAC in patients with "sporadic" CP is almost one order of magnitude lower [2]. This explains that only a fraction of patients with PDAC have a history of pancreatitis.

Modeling the role of pancreatitis in PDAC progression should be a bidirectional endeavor, whereby the knowledge acquired through studies with patients is tested in mice and the experimental studies should lead to novel hypotheses whose significance can be again tested in the context of the human disease.

Some of the main questions regarding the relationship between pancreatitis and PDAC are: (1) Does chronic inflammation cause mutations that contribute to PDAC? (2) Does pancreatitis act as a tumor-promoting event (i.e., through inflammation, oxidative stress, or genomic instability)? and (3) In which cells do the mutations that cause human PDAC arise? A fact that complicates some of these analyses is that PDAC itself can lead to a chronic pancreatitis-like lesion through duct obstruction, a situation that should be clearly distinguished from primary CP.

Animal Modeling of Pancreatitis

The anatomical location of the pancreas and the difficulties in obtaining tissue samples from patients, especially during the early stages of the disease, has underscored the need to develop animal models of pancreatitis. Currently, much of our understanding of the pathogenesis of acute and chronic pancreatitis comes from animal models. It is, however, important to remark that most of the experimental approaches used do not fully recapitulate the human disease, and the limitations of each of them need to be carefully considered. The very first experiments were

performed in 1856 by the physiologist Claude Bernard. Since then, many species have been used to model pancreatitis, including mice, rats, rabbits, cats, dogs, pigs, and opossum; however, rodents are most commonly used and standardized protocols have been established.

A first important consideration is that the human pancreas shows significant anatomical differences with respect to the pancreas of other species. The human pancreatic and bile ducts are separate and normally form a very short common channel in the duodenal wall, at the hepatopancreatic ampulla (ampulla of Vater), while in rodents, the two ducts fuse before they enter the duodenum, forming a long common duct that transports both bile and pancreatic juice. In the opossum, the bile and pancreatic ducts merge early and a long common duct drains into the duodenum.

Similar methods are used to induce experimental acute and chronic pancreatitis but, for the sake of clarity, they will be described separately.

Models of Acute Pancreatitis

In humans, approximately 80% of acute pancreatitis (AP) cases are related to either duct obstruction or ethanol abuse. However, only less than 10% of patients with gallstones or alcohol drinkers will develop pancreatitis [5]. Whether this reflects the contribution of additional factors or the severity of the primary cause is not well known. The complex etiology of acute pancreatitis has represented a challenge for the development of reliable animal models.

Animal models of AP can be classified based on the underlying strategy to mimic human disease etiology and pathophysiology: some of them aim at reproducing the putative triggering event (gallstone-dependent obstruction of the pancreatic duct, or excessive alcohol consumption), while others mimic the downstream biological processes (premature activation of pancreatic enzymes). Table 1 summarizes the characteristics of the main models used.

Obstructive Models

Mechanical approaches to mimic gallstone pancreatitis are largely based on the "common channel" theory proposed by Opie in 1901, whereby the presence of a stone in the ampulla of Vater would create a communication between the main pancreatic duct and the common bile duct, thus causing the retrograde reflux of bile in the former. Although many observations suggest that this theory is inaccurate, multiple models have been developed and are still used, which re-create the pancreatic duct obstruction, alone or in combination with bile reflux. These models include the closed duodenal loop, pancreatic duct ligation, and retrograde ductal infusion. All three techniques require surgery, involving anesthesia, and therefore necessitate skilled operators.

The **closed duodenal loop** protocol was first described in dogs [57] and consists of placing two ligatures upstream and downstream of the site of entry of the common bile duct and causing duodenal obstruction. This results in the reflux of the duodenal content into the biliopancreatic duct, which leads to AP. Because the procedure is

Table 1 List of the most common animal models used to study acute and chronic pancreatitis

Models of acute pancreatitis			
Type	Model	Species (most used)	References
Obstructive	Closed duodenal loop	Rat, dog	[6, 7]
	Duct ligation (pancreatic or biliary duct)	Rat, mouse, dog, rabbit, opossum	[8–11]
	Retrograde infusion	Rat, mouse, rabbit, dog	[12–15]
Nonobstructive	Caerulein administration	Mouse, rat	[16–23]
	Basic amino acids	Rat, mouse, rabbit	[24–27]
	Choline-deficient (CDE) diet	Mouse, hamster, cat, dog, monkey	[28]
	Alcohol administration	Rat, mouse, dog, cat	[29–32]
Models of chronic pancreatitis			
Type	Model	Species (most used)	References
Mechanical	Pancreatic duct ligation	Rat, mouse, dog	[33–35]
Chemical	Repeated caerulein administration	Mouse, rat	[36–44]
	Repeated L-arginin administration	Rat	[45]
	Prolonged CDE diet	Mouse	[46]
	Prolonged alcohol administration (combined with other triggers)	Rat, mouse	[47–51]
Genetic	*Cftr* inactivation	Mouse, pig	[52, 53]
	PRSS1 R122H mutant overexpression	Mouse	[54, 55]
	Spink3 inactivation	Mouse	[56]

difficult to standardize, there is wide variability in disease severity. This protocol has been adapted for rats [6], with the concomitant injection of either infected bile or combinations of bile salts and digestive enzymes, to increase damage. Infections are a major complication of this protocol, which is currently rarely used.

Duct ligation has been used in various species, including mice, rats, dogs, rabbits, and opossums. Ligation of the pancreatic duct alone is not sufficient to induce severe AP, with the interesting exception of the opossum, where the pancreatic and the biliary ducts merge early into a common duct, thus recreating the situation hypothesized by Opie in the common channel theory mentioned above [7]. Unfortunately, the opossum is not a particularly convenient species for experimentation, as animals need to be collected from the wilderness and there is a very high interindividual variation. Ligation of the common biliopancreatic duct leads to pancreatitis in rats [8], with multiple organ effects resembling the human disease. In mice and rats, bile duct ligation has been combined with bile infusion, or with stimulation of pancreatic secretion, in order to induce severe necrotizing pancreatitis [9, 10]. In addition to requiring surgical skills and to the variability associated with each individual procedure, other complications (e.g., peritoneal sepsis, duodenal wall necrosis) hamper the use of these models.

Retrograde infusion of bile acids, enterokinase, trypsin, or other digestive enzymes, into the pancreatic duct via the ampulla of Vater has also been used in

multiple species. The severity of the disease depends on the pharmacological agent administered, its concentration, volume, as well as on the pressure used for the infusion. Major limitations of this approach are the interindividual variability and the significant severity and mortality. The most common protocol consists of administering sodium taurocholate, a bile salt, into the pancreas of rats [11], and it has been adapted for rabbits, dogs, and, more recently, for mice [12]. In this procedure, a cannula is inserted in the pancreatic duct while the bile duct is clamped, to avoid that taurocholate reaches the liver. Taurocholate is infused through the cannula, which is then removed to allow the normal flow of bile and pancreatic juice. Immediately after infusion, hemorrhagic necrosis can be observed in the surrounding pancreatic parenchyma, which led to the hypothesis that taurocholate induces pancreatitis merely through its detergent activity. Nowadays it is known that multiple receptors are activated in response to taurocholate infusion, indicating broader mechanisms of action. In mice, administration of sodium taurocholate (50 μl of 2%) leads to hyperamylasemia at 24 h, as well as tissue edema and infiltration by neutrophils [12]. Higher concentrations of taurocholate cause more severe pancreatitis, with a 60% mortality rate at the highest dose used (5%), which is accompanied by systemic inflammation [13]. Notably, the ductal retrograde infusion method generally induces a severe hemorrhagic AP, which can even be lethal.

Nonobstructive Models

Caerulein is by far the most commonly used pharmacological agent to induce experimental AP. Caerulein is a peptide analog of the gastrointestinal hormone cholecystokinin (CKK), which was originally extracted from the skin of the Australian green tree frog (*Litoria caerulea*). There are two types of CCK receptors, CCK1R (alimentary, also known as CCK-A) and CCK2R (brain, also known as CCK-B), with species-specific cell and tissue distribution [14]. CCKR belong to the family of G protein-coupled membrane receptors and therefore signal through the activation of multiple downstream pathways including phospholipase C, phosphoinostide 3 kinase, and MAP kinase. The CCK1R receptor binds preferentially the sulfated form of CCK, while CCK2R can bind either sulfated or non-sulfated CCK, as well as gastrin, with similar affinity [15]. CCK1R is the major receptor responsible for CCK-induced secretion of pancreatic enzymes [16]. The expression, localization, and function of CCK1R and CCK2R in the human pancreas are still under investigation. There is no conclusive evidence that human acinar cells display a functional response to CCK or gastrin or that they express CCKR [58]. There are substantial differences in receptor expression among species, as well as controversy on their function.

In rodents, physiological plasma levels of CCK (or caerulein) bind to the high-affinity receptors and evoke production and secretion of pancreatic enzymes. Supramaximal concentrations of either CCK or caerulein engage the low-affinity receptors, which block exocytosis [14]. This causes the accumulation and abnormal subcellular distribution of the digestive proenzymes, mainly trypsinogen, with premature fusion of zymogen granules with lysosomes, leading to their activation within the acinar cells. Cathepsin B has been proposed to play a key role in this

process [59]. The consequence is tissue damage, due to an autodigestive process, followed by edema and inflammatory cell infiltration, thus recapitulating a mild, transient, and self-limiting acute pancreatitis.

Caerulein-induced AP has been extensively used in mice and rats, with multiple protocol variants. In rats, caerulein is typically administered intravenously (i.v.) either as a bolus or by continuous i.v. infusion. In mice, intraperitoneal (i.p.) administration has been preferred, usually through multiple (4–12) hourly injections. A very common protocol to induce a mild edematous AP with caerulein in mice includes 7-hourly administration of the drug at 50 μg/kg [60]. However, in recent years, a wider variety of protocols has been applied, mainly in combination with genetically engineered mouse models (GEMMs) of pancreatic disease. A common variant implies the administration of two rounds of 6–7 hourly doses, either on two consecutive days or on days 1 and 3 [17]. As described below, it is important to distinguish between the 1-day and 2-day protocols because they generate significantly different extents of tissue damage, followed by tissue remodeling and regeneration, and they might even engage distinct cellular/molecular processes [18]. Molecular signaling events activated by caerulein are induced as early as 30–60 min after the first injection. These include the activation of the MAPK cascade, activation of the PI3K pathway, and the consequent induction of NF-κB, as well as the Stat3 pathway. Thereafter, the single-day protocol causes extensive edema, infiltration by inflammatory cells, and acinar cell vacuolization and collapse, with a peak around 24 h from the first injection and full recovery by day 7. The 2-day protocol, particularly on consecutive days, results in extensive loss of the acinar cell compartment, caused by massive cell dedifferentiation and tissue remodeling through a transient acinar-to-ductal metaplasia (ADM), whereby acinar cells rapidly lose the expression of acinar markers and ectopically express markers of the ductal lineage (see also Sect. 5.1 below) [18, 19, 60]. The 2 consecutive-day protocol has a low lethality rate and animals that survive treatment display complete recovery within 7–14 days, similar to the 1-day protocol.

In most of the experiments described above, pancreatitis was induced in young mice (around 1.5–2 months). Interestingly, Okamura et al. have shown that the multidose 1-day caerulein acute pancreatitis is significantly more severe in older (23–25 months) mice, as evidenced by increased plasma amylase and Il-6 levels at 12–24 h, higher neutrophil infiltration, and more severe extrapancreatic tissue damage, including evidence for disseminated intravascular clotting and extensive fibrin deposition in the lung and kidney of old mice [20].

Caerulein-induced AP models have several advantages, as they are simple, reproducible, inexpensive, and noninvasive. They can also be paralleled with ex-vivo studies using primary acinar cell cultures, which are extremely useful to acquire mechanistic insights. However, their relevance to the human disease has been questioned. Although Mouret reported already in 1895 that excessive cholinergic stimulation produced vacuolization and necrosis of pancreatic acinar cells in dogs, and proposed that activation of trypsin could be involved in the process, the supramaximal stimulation of the human pancreas has been reported only very rarely. Pancreatitis was reported in patients after intoxication with anticholinesterase-

containing insecticides and after exposure to the acetylcholine-inducing scorpion venom. In all these cases, excessive amounts of acetylcholine are released by pancreatic nerves, resulting in hyperstimulation of enzyme secretion, often leading to uncontrolled and premature activation of the digestive enzymes within the tissue. Furthermore, most of the genetic alterations that have been associated with increased risk of CP involve genes coding for proteins involved in the activation of the digestive enzymes, or their inhibitors [21], supporting the use of caerulein to study the mechanistic events underlying pancreatitis, regardless its relevance as potential cause of AP.

On the other hand, caerulein induces in rodents a regenerative/inflammatory pancreatitis that possibly recapitulates the milder forms of disease in humans, rather than the severe necrotizing disease that is more clinically worrisome and life-threatening. Finally, there are major differences between humans and rodents regarding the mechanisms of stimulation of secretion.

Basic amino acids, such as L-arginine, L-ornithine, and L-lysine, administered i.p. at high doses cause selective damage to acinar cells in rats, rabbits, and mice, resulting in an acute necrotizing pancreatitis associated with a strong inflammatory reaction [22, 23]. A variable fraction of the animals succumb within the first 48 h due to poorly characterized, pancreatitis-unrelated, reasons. Animals that survive display complete recovery by day 14. The marked histological selectivity for acinar cells is reminiscent of human necrotizing pancreatitis, where nerves, major ducts, and islets are not affected but, unlike in patients, systemic complications are rare. The mechanism of induction of pancreatitis is not known. It has been proposed that inhibition of protein synthesis, excessive nitric oxide production, or increased lipid peroxidation play a role [61, 62].

Choline-deficient, ethionine-supplemented (CDE) diet is the least invasive method to induce AP, since it only requires a change in the diet. Administration of a CDE diet for a short time (2–5 days) has been shown to induce pancreatitis in multiple species including mice, hamsters, cats, dogs, and monkeys. In female mice, CDE diet induces an acute hemorrhagic pancreatitis with fat necrosis and prominent liver injury already after 3 days, resulting in high mortality. Male mice are resistant, unless estrogen is administered, suggesting the involvement of sex hormones in the response [24]. The exact mechanisms through which CDE diet induces pancreatitis are not known. Importantly, the CDE diet-induced pancreatitis shares a number of features with the human disease, including the histological appearance of the pancreatic and peripancreatic inflammation, the clinical and biochemical course, necrosis, and systemic hypoxia. Adjusting the duration of the diet can modify the severity and mortality of this model. However, implementation of the CDE diet might be troublesome, because animals tend to dislike it and a careful monitoring of the dietary intake is necessary.

Alcohol administration has also been extensively used to induce AP, based on the established epidemiological association between AP and alcohol consumption in patients. However, acute or chronic administration of alcohol alone fails to induce pancreatitis in all of the species used. Multiple studies where ethanol was administered either acutely or chronically through different routes (intravenous, oral, or

intragastric) showed that alcohol can enhance the acute and chronic pancreatitis induced by other experimental manipulations, including secretagogue or lipopolysaccharide (LPS) administration, high-fat diet, or surgical intervention [25, 26]. It is possible that the failure to reproducibly induce pancreatitis with ethanol alone in experimental models reflects the fact that alcoholic pancreatitis is a multifactorial disease, whereby other lifestyle and environmental factors (i.e., smoking, obesity), as well as genetic predisposition, play a role [27].

Models of Chronic Pancreatitis

Chronic pancreatitis is currently thought to result from recurrent AP. It involves continuous or recurrent damage/inflammation of the pancreatic parenchyma, which undergoes progressive and morphological and histological changes including loss of exocrine and endocrine mass, fat replacement, inflammatory cell infiltration, necrosis, stellate cell activation, fibrosis, calcification, and nerve enlargement. The notion that the pathological features of CP are irreversible is currently being challenged and the hypothesis that irreversibility might be the consequence of late diagnosis is under examination [1]. Animal models of CP have been developed which, however, do not fully reproduce the clinical presentation and clinical course of the human disease. This may be related to the fact that in humans, the diagnosis of CP is commonly made late during disease progression, but it may also reflect important species-specific pathogenic responses.

Two theories have been proposed on how CP develops in patients: as a result of multiple bouts of AP (either subclinical or clinically evident) or through a single severe initiating event whose effects are prolonged and sustained until they become irreversible. Experimental models suggest that both mechanisms can indeed lead to CP. Three major types of experimental CP have been used: ***mechanical*** (e.g., duct ligation) – mimicking the obstructive lesions that are associated with CP in patients; ***chemical*** (e.g., caerulein, L-arginine, or ethanol administration), reproducing the downstream mechanisms of injury or relating to the human CP etiology; and ***genetic,*** based on the germline mutations identified in humans. Importantly, a general feature of all mouse models of CP is the lack of the massive fibrosis that is observed in patients, possibly indicating species- or strain-specific biological differences.

Pancreatic duct ligation (PDL) (partial, selective, or complete) has been used for the induction of CP in dogs, rats, and mice, with markedly species-dependent outcomes. This approach aims to mimic the obstruction caused by protein plugs in the small pancreatic ducts of CP patients, which cause increased retrograde pressure. In mice, ductal ligation induces a massive loss of acinar cells that are replaced by adipocytes, consistent with clinical-pathological observations in humans. PDL is often combined with the administration of ethanol or caerulein [63]. A modification of this method is the pancreatic duct hypertension procedure [28], developed for rats, where hydrostatic pressure is exerted in the pancreatic duct, mimicking the pressure resulting from duct obstruction. This method is technically challenging and has not been adapted for mice.

Caerulein, administered repeatedly over several weeks, is probably the most commonly used pharmacological agent used to induce CP, especially in studies assessing the relation between inflammation and cancer [29]. This approach is based on the clinical observation that chronic ethanol consumption and repeated events of AP can lead to CP. Although single AP events are fully self-limiting and reversible, as mentioned above, repetitive insults eventually result in chronic inflammation, acinar cell atrophy, ADM, fibrosis, and – in extreme cases – diabetes. In rats, these effects are partially reversible upon discontinuation of caerulein administration [30]. It remains to be determined whether the same is true in mice.

The amount, frequency, and total duration of caerulein administered differ substantially among studies and the optimal schedule is far from being standardized. Protocols used include full AP induction, i.e., 6–7 hourly injections of caerulein during 1 day, two or three times per week, over 6–10 weeks [31, 32, 64], or are limited to single daily injections, 5 days per week, over 10–12 months [65]. Severity and kinetics of disease progression can therefore vary widely and a systematic comparison of these protocols has not been reported. Caerulein is often used in combination with other pharmacological agents, such as LPS, a bacterial endotoxin that can activate stellate cells and stimulate inflammation, thus increasing the extent of fibrosis induced by caerulein and accelerating the disease in mice [33]. Cyclosporine was also used to exacerbate the chronic inflammation induced by caerulein in rats [34]. Finally, caerulein administration has been combined with PDL in rats, where it induces CP with necrosis followed by fibrosis, and in mice.

Repeated administration of **L-arginine** and chronic administration of the **CDE diet** has also been used to induce CP. Serial injections of L-arginine to rats induce severe acute pancreatitis, which progressively results in chronic damage, characterized by persistent acinar cell atrophy and fat replacement, occasionally accompanied by necrosis and fibrosis [35]. On the other hand, mice intermittently fed with the CDE diet for a prolonged period of time (up to 54 weeks), develop acinar atrophy, ADM, and a mild fibrosis [36].

Alcohol abuse is among the major causes of CP in patients but, as mentioned above for AP, the many models of alcohol-induced pancreatitis that have been explored have not successfully recapitulated the human disease, so far. This is consistent with the fact that less than 10% of alcoholics develop CP, despite regular excessive alcohol consumption. Ethanol supplementation to the diet for up to 6 months causes a partial exocrine impairment in rats, but no morphological changes characteristic of CP are observed [37]. Administration of higher doses of ethanol with the Lieber–DeCarli liquid diet [38] fails to induce severe damage to the pancreas, but it sensitizes cells to other triggers, such as caerulein [39] or LPS [40], although the resulting disease resembles more acute than chronic pancreatitis. CP can be successfully and consistently induced in rats when alcohol is administered through intragastric infusion in gradually increasing doses, in combination with a diet rich in unsaturated fat [41]. This protocol produces hyperamylasemia and hyperlipasemia, as well as acinar cell atrophy, inflammatory cell infiltration, and focal necrosis. Longer treatment also induces some extent of fibrosis. Disease development is paralleled by increased levels of free radicals within the pancreas,

supporting the hypothesis that oxidative stress is at the basis of alcohol-induced pancreatitis. This model suggests that the total amount of alcohol consumed and the type of dietary fat that is ingested are crucial determinants of CP [41].

Genetically engineered animal models (mostly mice, with few exceptions) have been developed, aiming at recapitulating the genetic alterations that have been associated with higher risk of CP in patients. These models have high clinical relevance but the extent of their similarity to the human disease varies considerably. For example, inactivating mutations of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene cause cystic fibrosis, a hereditary disease of chloride ion channel, which is associated with lung, intestinal, and pancreatic disease including a chronic pancreatitis-like lesion and increased risk of cancers of the digestive tract [42]. *Cftr* inactivation in mice produces only mild pancreatitis [43], while its inactivation in pigs causes rapid progressive pancreatic disease similar to what is observed in patients with cystic fibrosis [44].

One of the putative triggers for pancreatitis is the premature, intracellular activation of trypsinogen [45]. The design of GEMMs aimed at recapitulating these alterations is complicated by the existence of multiple genes coding for trypsinogens in human and mice and lack of knowledge about which are the most appropriate orthologues. Germline mutations in *PRSS1*, most notably the R122H mutation, are strongly associated with autosomal dominant hereditary CP [46]. When a mutant mouse cDNA harboring an equivalent mutation was expressed in the pancreas of transgenic mice, fibrosis and acinar cell dedifferentiation consistent with CP were observed [47], while the ectopic expression of the human mutant cDNA did not induce any histological change [48]. In both cases, expression of mutant Prss1 rendered animals more susceptible to caerulein-induced pancreatitis [47, 48]. Mutations in other genes whose products are involved in enzyme secretion and activity, such the serine protease inhibitor Kazal type 1 (*SPINK1*), chymotrypsinogen C (*CTRC*), and the calcium-sensing receptor (*CASR*) have also been associated with increased risk of pancreatitis [49]. Deletion of *Spink3*, the mouse homologue of *SPINK1*, causes elevated trypsin levels followed by massive autophagy in acinar cells, which is lethal within 2 weeks from birth [50]. Currently, no genetically engineered mouse model is available, expressing mutations in *Ctrc* or *Casr*.

Genetic Models of Pancreatic Inflammation

Several GEMMs have been reported, displaying spontaneous pancreatic damage mimicking acute or chronic pancreatitis. For example, the overexpression of the proinflammatory cytokine Il-1β in acinar cells is sufficient to induce CP in mice, with the first signs of inflammation starting already at 1 week and progressing with age [51]. Similarly, overexpression of Cox2, a molecule that is activated by inflammatory cytokines, under the control of the *Krt5* promoter, also induces CP and even some ductal neoplastic lesions [66]. NF-κB is activated in acinar cells during the early stages of experimental pancreatitis [67], therefore multiple models have been developed which induce the activation of the NF-κB pathway. These models,

however, reveal a more complex role of this pathway, since its activation can lead to both aggravation [52] and amelioration of pancreatitis [53], due to the known dual role of NF-κB, which can be both pro- and anti-inflammatory. Furthermore, members of the NF-κB pathway seem to also have a NF-κB-independent function in protecting acinar cells from endoplasmic reticulum (ER) stress and autophagy [68]. Finally, mice lacking the essential autophagy-related proteins Atg5 [69] and Atg7 in pancreatic cells also develop a pancreatitis-like phenotype, due to ER stress [70], suggesting a more complex mechanism than induction of autophagy (see below).

Somatic Genetic Alterations in Chronic Pancreatitis

Chronic pancreatitis is characterized by ADM, acinar atrophy (focal or diffuse), ductal stasis, and all types of pancreatic intraepithelial neoplasia (PanIN), as well as mesenchymal cell proliferation and activation, extracellular matrix (ECM) deposition with collagen accumulation, and inflammatory cell infiltration.

Experimental and molecular pathology evidences suggest that ADM and PanINs can be the precursors of PDAC and at least two-thirds of patients with CP undergoing surgery have PanINs, their prevalence decreasing with increasing dysplasia [54].

Accordingly, it is conceivable that some of the genetic alterations characteristic of PDAC would be present in epithelial lesions associated with CP. Because >95% of PDAC harbor *KRAS* mutations and because of the proposed tumor-initiating role, these mutations are top candidates for being present in CP tissue samples. However, the cause of *KRAS* mutations in PDAC is not known and there is not clear evidence that mutations are linked to the most common risk factors for PDAC. Overexpression of Ikk2 or Cox2 in p53-null acinar cells causes tumors that are *KRas*-wild type, suggesting that chronic inflammation does not efficiently cause *KRas* mutations in mice [55]. It is likely that the almost universal occurrence of mutant *KRAS* in PDAC reflects the exquisite sensitivity of pancreatic cells to this oncogene rather than the action of a single specific mutagenic event that could be used to model their appearance in experimental animals. Several studies have assessed the prevalence of *KRAS* mutations in samples from patients with CP (pancreatic/duodenal juice, endoscopic ultrasound-guided fine needle aspiration (EUS-FNA), pancreatic tissue, or plasma). In all cases, the prevalence of *KRAS* mutations was lower than in patients with PDAC, ranging from 11–37% in juice, being lower in EUS-FNA or in tissue, and essentially undetectable in plasma [56, 71–73].

The tumor suppressor genes *CDKN2A* (coding for $p16^{INK4A}$), *TP53*, and *SMAD4* are inactivated in approximately 90%, 50–75%, and 60% of PDAC cases, respectively, through mutation, genomic loss, or promoter hypermethylation [74]. There is less information regarding alterations in these tumor suppressors in CP tissue, in part due to the limitations derived from tissue sampling, histopathological heterogeneity, and sensitivity of the techniques used. Heterozygous mutations in $p16^{INK4A}$ and

TP53 have been detected in a low fraction of PanIN and ADM lesions from patients with CP without cancer; mutations were homozygous in PanIN-3 but not in low-grade PanINs, suggesting clonal evolution associated with lesional progression [71]. The prevalence of *KRAS* and *TP53* mutations, and of $p16^{INK4A}$ methylation, in pancreatic juice was intermediate in CP compared to PDAC and control samples [73]. Aberrant gene methylation was found to be higher in pancreatic juice or tissue from patients with CP than in controls, although it was lower than in patients with PDAC (it was similar to high-risk individuals), suggesting that epigenetic mechanisms contribute to the progression of CP to PDAC [56, 72]. Using immunohistochemistry, $p16^{INK4A}$ – but not SMAD4 – has been found to be lost only in PanIN-2/3 [56, 75].

So far, there have been no reports on the genetic landscape of somatic mutations in CP samples using massive parallel sequencing, or the more recent techniques with a high sensitivity to detect gene mutations (i.e., digital PCR, Beaming), to shed light on the genetic events that precede PDAC development in patients with CP. It will be important to determine whether distinct genetic/genomic alterations characterize the CP-associated tumors.

In summary, there is strong evidence that the pancreas of patients with CP harbors genetic alterations that are characteristic of invasive PDAC, albeit at lower frequency but much work remains to be done in this domain.

Modeling of the Contribution of Pancreatitis to Cancer in Mice

Most of the experimental information gathered in the last 15 years on the contribution of CP to PDAC has been acquired using GEMMs. This has been possible thanks to the fact that PDAC is one of the human tumors that has been best recapitulated using GEMMs in which the main PDAC-associated mutations have been introduced.

Current evidence indicates that mutations in *KRAS* oncogene are the main, if not exclusive, oncogenic event leading to PDAC [76]. Therefore, the best models to assess the contribution of pancreatitis to cancer are those in which mutant KRas is conditionally expressed in the pancreas through the activation of Cre recombinase in specific cell populations. The second main oncogene involved in PDAC is *GNAS*. Hotspot codon 201 mutations have been described in a variety of human tumors; in the pancreas, *GNAS* mutations occur mainly in IPMNs and most of the PDAC harboring these mutations result from the progression of IPMN precursor lesions [77, 78]. *GNAS* functions downstream of G-coupled membrane receptors and inhibits the activation of adenylyl cyclase which, in turn, raises cAMP levels. Mice expressing both mutant KRas and mutant Gnas in the pancreatic lineage develop tumors reminiscent of IPMN [79]. Recently, induction of chronic inflammation by overexpression of the p65 subunit of the inhibitor of NF-kB kinase (Ikk2) or Cox2 in acinar cells, together with the deletion of *Trp53*, has been shown to lead to the formation of *KRas*-wild type (WT) tumors displaying a wide variety of histologies (acinar, ductal, sarcomatoid, neuroendocrine) [55].

The Conditional Mutant *KRas* Mouse Models: Twenty-First Century Tools

Studies in the second half of the twentieth century aimed at developing animal models of pancreatitis and pancreatic cancer, used predominantly chemical carcinogenesis, mainly in mice, rats, and hamsters [80]. Many of the models used yielded acinar rather than ductal tumors, and these studies rarely addressed the pathophysiological and molecular relationship between both tumor histologies and the causes for the species-specific differences.

Since 2003, two main conditional GEMMs have been used to activate expression of mutant *KRas* in the pancreas, based on either the G12D [81] or the G12V [65] codon 12 mutation. In most studies, expression was activated during embryonic development in multipotent pancreatic progenitors. Using the G12D mutant and a $Pdx1^{Cre}$ driver strain (KC mice), PanIN-1 and PanIN-2 lesions are detected by weeks 10 and 20–24, respectively. PanIN-3 are exceptional in young mice and only appear later. In older mice, PDAC develops and causes the death of 50% of mice by 12 months age. The simultaneous introduction of a *Trp53* mutation in this strain (KPC mice) leads to faster tumor initiation and progression, with frequent development of metastases; median survival is 6 months and no mice are alive by 12 months age [82]. Similarly, simultaneous activation of mutant *KRas* and inactivation of both alleles of $p16^{Ink4a}$/$p19^{Arf}$ leads to very aggressive tumors with anaplastic/sarcomatoid features and to the death of all mice by 16 weeks [83]. When *KRas* mutations and *Smad4* inactivation are introduced simultaneously, all mice have tumors by 6 months and the histology is often reminiscent of IPMNs [84]. In summary, the cooperation of *KRas* mutations with other genetic alterations common in PDAC has been clearly demonstrated.

Using the G12V conditional allele, Guerra et al. have confirmed the ability of mutant *KRas* to induce PanINs and PDAC when activated in acinar cells during embryonic development using a doxycycline inducible strategy to express Cre recombinase (e16.5). In this model, PanIN-1 lesions are focally detectable in the majority of 3-month-old mice and by 6 months, most mice have acquired multiple PanIN-1 lesions diffusely, some PanIN-3, and occasional PDAC. By 1 year, the majority of the mice have PanIN-1 lesions, 35% have high-grade PanINs, 50% of mice have PDAC, and 12% of them have died [65]. PanINs and PDAC are commonly embedded in a desmoplastic microenvironment containing abundant collagen fibers and fibroblasts, as is characteristic of the lesions found in the pancreas of patients with PDAC. Notably, several important observations have been made using this strain (Fig. 1). First, it was shown for the first time – using lineage tracing – that PDAC can arise from acinar cells in which the expression of the mutant *KRas* oncogene is selectively activated, raising the question of the cell of origin of human PDAC. Second, when mutant *KRas* expression was activated in young adult mice (2 months), no PanINs or tumors developed by 1 year, indicating that – in homeostatic conditions – adult acinar cells are largely refractory to the oncogenic effects of mutant KRas. Importantly, daily administration of a single dose of caerulein to mice in which the mutant *KRas* allele is activated at the age of 2 months leads to the rapid development of low-grade PanINs (within 1 month), increased acinar cell

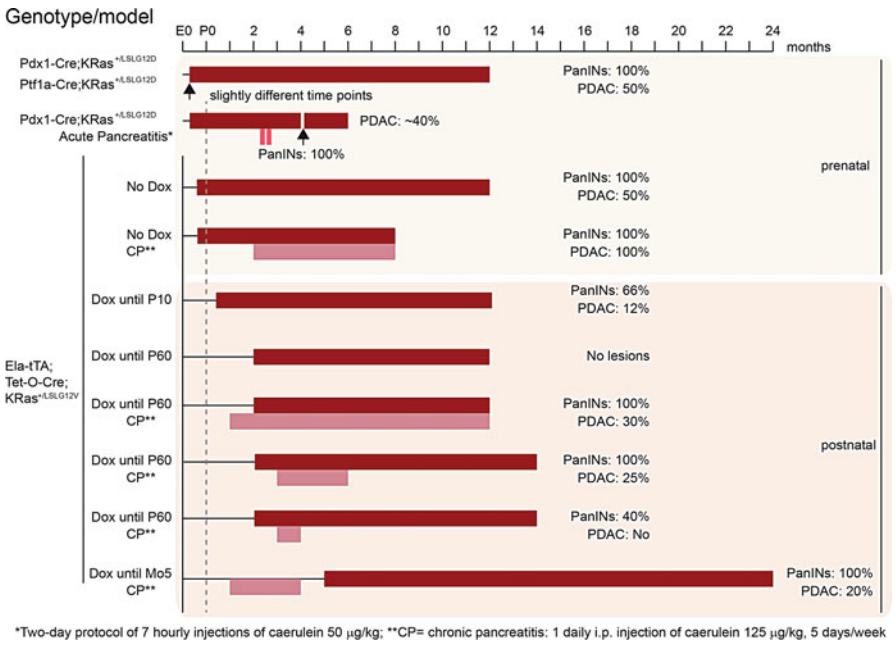

Fig. 1 Main *KRas*-mutant GEMM and protocols of acute and chronic pancreatitis used to study the pancreatitis-to-PDAC progression process

proliferation, and inflammatory cell infiltration. By 8 months, an increasing number of low-grade and high-grade PanINs is observed and one-third of the mice have developed invasive PDAC. A similar enhancement of the tumorigenic effect of mutant *KRas* by chronic caerulein administration is observed when the oncogene is activated during embryo development [29]. The persistence and the extent of the caerulein-induced damage are important variables and longer caerulein treatment is associated with a higher prevalence of PanINs and PDAC or a shorter latency of lesion development (Fig. 1). Caerulein administration is associated with increased inflammatory infiltrates in which neutrophils and eosinophils predominate in a first phase, subsequently containing a more complex constellation of cell types (macrophages, B and T cells, and plasma cells) (see below).

An additional important finding made using this strain was the fact that adult acinar cells were resistant to mutant KRas-mediated transformation, in the absence of pancreatitis, even when *Trp53* or $p16^{Ink4a}$ were simultaneously inactivated in the same cells of the adult pancreas. By contrast, deletion of these tumor suppressors effectively cooperates with mutant *KRas* when activated during embryo development. Inactivation of the tumor suppressors *Trp53* or $p16^{Ink4a}$ in adult mice never leads to PanIN or PDAC in the absence of mutant *KRas*, even when caerulein is administered, strongly supporting the initiating nature of this mutation [29]. Administration of caerulein over 3 months to young mice, and subsequent activation of the *KRas* oncogene, also leads to the appearance of PanINs and PDAC [29]. These results are

in agreement with the notion that chronic inflammation does not efficiently cause *KRas* mutations and point to the relevance of the sequence of events, suggesting that preexisting damage sensitizes acinar cells to the effects of mutant KRas.

Altogether, there is compelling evidence supporting the usefulness of GEMMs to model and recapitulate the interaction of genetic (somatic mutation) and nongenetic (epithelial cell remodeling and inflammation) factors driving PDAC development, as occurs in patients.

Acute Pancreatitis-Associated Damage Promotes PDAC

A question raised by the studies described above was whether more limited damage could also enhance the oncogenic effects of mutant *KRas*. Carriere et al. were first to show, using 2-month-old mice in which mutant *KRas* had been activated in pancreatic progenitors, that a 2-day caerulein acute pancreatitis is sufficient to enhance PanIN development and accelerate mutant KRas-driven PDAC progression [17]. This effect was confirmed in several additional studies [85, 86]. Similar results were obtained when mutant *KRas* was activated only in nestin-expressing cells [87]. In these experiments, pancreatitis was resolved within 1 week, when inflammatory infiltrates had almost completely disappeared but increased acinar Ki67 expression was noted. This was followed by extensive replacement of the acinar parenchyma by ADM, low-grade and high-grade PanIN lesions, with later development of PDAC. Subsequent to this work, it has been shown that the mildest form of acute pancreatitis – resulting from 7-hourly injections of caerulein – also accelerates PanIN formation and PDAC development in a context in which mutant *KRas* is concomitantly expressed in the pancreas [88]. As will be discussed below, these studies strongly suggest that perturbation of acinar cell homeostasis for a brief period of time also sensitizes pancreatic cells to mutant KRas (Fig. 1).

Regarding humans, there is little epidemiological evidence that past medical history of a single episode of AP is associated with an increased risk of developing PDAC. Recently, the occurrence of AP in the months preceding the diagnosis of PDAC has been reported but it is not known whether such event simply heralds the development of the tumor or it could, in fact, act to promote its progression [89] and further work in this area is warranted.

Developmental and Cellular Mechanisms Involved in CP-to-PDAC Progression

The cellular mechanisms underlying the tumor-promoting effect of both acute and chronic pancreatitis are still under investigation. The GEMMs have pointed to the contribution of both cell autonomous and non-cell–autonomous events. Epithelial cell-autonomous processes include tissue regeneration, acinar cell proliferation, and escape from senescence. Additionally, an important role of nonepithelial cells such as pancreatic stellate cells (PSC) and inflammatory infiltrates has been demonstrated.

Epithelial Cell-Autonomous Mechanisms

Dedifferentiation of Acinar Cells and ADM

The experimental models of acute and chronic pancreatitis described above have unveiled the remarkable plasticity of the pancreatic parenchyma, particularly of acinar cells. As mentioned above, one single episode of caerulein-induced AP is sufficient to induce a drastic loss of the exocrine function within the first 24 h; however, the pancreas is histologically back to normal already after 5–7 days. Transcription of acinar-specific genes, such as amylase, is rapidly downregulated to be restored almost to baseline levels after 7 days [18]. Jensen and colleagues showed, in a comprehensive analysis of tissue regeneration after AP, that acinar cells rapidly silence the expression of the acinar differentiation marker amylase. This is followed by the induction of Pdx1, a major driver of pancreatic development, which in the adult is expressed at high levels only in endocrine cells, while expression in exocrine cells is very low. Rapid activation of the Notch pathway is also observed in this model, suggesting a broad reactivation of transcriptional programs that are normally restricted to embryonic development [18]. Extensive data currently support the notion that during AP, acinar cells lose their identity, reexpress markers of multipotent progenitors, including Pdx1, Sox9 [85], and Nestin [19], and reactivate developmental programs, such as Notch, Hedgehog, and Wnt pathways, which are thought to be essential for the efficient regeneration of the tissue. More in detail, expression of genes involved in the Notch pathway (i.e., Notch1, Dll1, Rbp-jk, and Hes1) is induced after caerulein-induced pancreatitis in mice [18], and pharmacologic or genetic ablation of Notch signaling results in strongly impaired regeneration after AP [90]. Also the Hedgehog pathway is extensively induced after caerulein-induced AP, where the expression of Hedgehog ligands Shh and Ihh, as well as the receptor Smo and the Hedgehog-regulated genes Ptch1 and Gli1, are strongly upregulated during the regenerative phase. Also in this case, pharmacologic blockade or genetic ablation of signaling components impairs tissue regeneration [19]. Strong induction of β-catenin, the prime transcriptional activator of the canonical Wnt pathway, takes place in regenerating acini after caerulein-induced pancreatitis, with a predominant accumulation at the cell periphery and in the cytoplasm, corresponding with a general activation of canonical Wnt signaling [18, 85]. Genetic ablation of β-catenin impairs the regeneration of pancreatic tissue after pancreatitis [85].

In this line, a recent study by Kong and colleagues has compared the dynamics of AP in WT mice and in mice expressing mutant KRas and shown that the response of the WT pancreas to caerulein-induced AP (2-day protocol) includes three phases: (i) acute inflammation, characterized by edema, immune infiltration, and ADM; (ii) regeneration, during which immune cell infiltration and ADM gradually disappear; and (iii) refinement, during which the tissue recovers completely. In the presence of mutant KRas, this sequence is perturbed, and the initial phase of inflammation does not resolve [91]. An important event during the acute inflammatory phase in both WT and mutant KRas-bearing mice is the rapid inhibition of a transcriptional program linked to acinar cell homeostasis, which is only reactivated

during the regeneration phase in the WT pancreas. This transcriptional program is strongly downregulated in PDAC cells suggesting that its inhibition favors tumor development.

Likewise, dedifferentiation of acinar cells has been reported in two distinct models of CP in mice, namely PDL and caerulein administration, in association with the upregulation of the progenitor markers Pdx1, Sox9, Hfn1b, and Hes1 [92]. Some differences in gene and protein expression exist between the two protocols, suggesting that the extent of tissue damage might influence the amplitude of the response [92].

It was mentioned above that adult acinar cells are refractory to transformation induced by mutant KRas, while pancreatic multipotent progenitors as well as unipotent acinar progenitors are susceptible. The observation that acute and chronic pancreatitis produce a transient dedifferentiation of acinar cells towards a progenitor-like phenotype might support the hypothesis that pancreatitis favors PDAC development through the transient expansion of the pool of cells sensitive to mutant KRas, thereby increasing the probability that at least one of the cells targeted by mutant KRas progresses towards PanIN and PDAC. This notion would be consistent with the observation that loss of major drivers of acinar cell differentiation and maintenance, such as Ptf1a, Gata6, Nr5a2, and Mist1 – alone or in combination with pancreatitis – significantly accelerates the development of tumors in the presence of mutant KRas [88, 93–95].

The transiently dedifferentiated acinar cells observed after pancreatitis are not identical to the pancreatic progenitors present during development, as they additionally express some markers of ductal cells, such as Krt19, revealing a process of ADM [85]. ADM was suggested to be a precursor of PanINs and PDAC and is therefore considered one of the first events induced by mutant KRas during tumorigenesis. Indeed, expression of mutant KRas on its own is sufficient to induce an ADM that closely resembles the phenotype shift induced by pancreatitis [96]. Importantly, although some differences have been observed in the ADM-inducing mechanisms between mouse and human acinar cells, ADM takes place in human primary acinar cells upon treatment with TGF-β in vitro [97] and it can even occur spontaneously [98] indicating that it is a biologically relevant process and that the acinar program is sustained actively. Therefore, it is conceivable that the pancreatitis-induced ADM generates a tumor-competent environment, which facilitates cell transformation driven by oncogenic KRas.

Pancreatitis and mutant KRas activate similar transcriptional programs to induce dedifferentiation and ADM, involving transcription factors such as Sox9, Myc, Klf4, and Pdx1. However, ADM becomes irreversible and progresses towards PanINs and PDAC only in the presence of mutant KRas. This might not reflect the simple additive effect of similar ADM-inducing signals originating from pancreatitis and oncogenic KRas, as shown by the observation that the initial histological and transcriptional patterns are similar in mice harboring the mutant oncogene and in WT mice [85, 91], but more complex mechanisms might be involved. The major consequence of *KRas* mutation is that acinar cells are locked in a dedifferentiated state, and tissue regeneration is inhibited due to a failure to transiently induce β-catenin and activate the Wnt pathway [85]. The stronger and persistent activation

of the MAPK pathway (and possibly other signaling pathways) in the presence of mutant KRas may be the major underlying cause [85]. Kong et al. have identified a complex network of molecular interactions that is responsible for tissue regeneration in WT mice, involving both intrinsic and extrinsic cues. Interestingly, this network is very much simplified in the presence of mutant KRas, thus impairing the proper resolution of tissue damage and favoring tumor initiation [91].

Acinar Cell Proliferation

Tissue regeneration after AP is associated with a peak of proliferation of acinar cells, which are normally quiescent. In a model of AP where mice receive 8-hourly injections of caerulein over two consecutive days, the proportion of acinar cells expressing phosphorylated histone H3, a marker of mitotic cells, increases 40-fold 3 days after caerulein injection and then gradually decreases to baseline after 7 days [18]. Similar findings have been made using BrdU uptake in a mouse model of AP that used the 1-day caerulein protocol, where the peak in acinar cell proliferation occurs 4 days after injection [60]. Interestingly, increased proliferation of interstitial cells, mainly myeloid cells, takes place as early as 7 h after the first injection of caerulein, again pointing to an orchestrated response involving both epithelial and stromal cells.

Hyperproliferation of acinar cells might contribute to the protumorigenic effect of pancreatitis through an increase in DNA replication, which provides an opportunity for errors and disease-causing mutations. Interestingly, however, two distinct cell populations displaying opposite phenotypes have been identified in two models of CP; while some acinar cells express proliferation markers, another subpopulation expresses markers of senescence, suggesting that a protective mechanism against the potential danger of uncontrolled proliferation is activated during tissue regeneration following damage [92]. Whether a subpopulation of progenitor-like adult acinar cells that become active after tissue injury participates in regeneration needs to be definitely ruled out. Lineage tracing experiments strongly support the notion that all acinar cells have similar potential to reenter the cell cycle and divide during tissue regeneration [99] but recent work points to the existence of a previously undescribed heterogeneity of adult acinar cells both in murine and human pancreas [100].

Inhibition of Oncogene-Induced Senescence

Another cellular mechanism involved in the protumorigenic effects of pancreatitis is the inhibition of oncogene-induced senescence in the early stages of PanIN progression [29]. Hyperactivation of oncogenes in normal cells can cause senescence, mediated by the $p16^{Ink4a}/p19^{Arf}$ or *TP53* tumor suppressors, as a mechanism of defense against malignant transformation [101]. Markers of senescence have been detected in ADM and early PanINs in two distinct mouse models of PDAC induced by mutant KRas but not in high-grade PanINs and PDAC [102, 103]. Based on this observation, Guerra and colleagues hypothesized that adult acinar cells are refractory to transformation induced by oncogenic KRas due to the activation of cellular senescence. Genetic ablation of either $p16^{Ink4a}/p19^{Arf}$ or *Trp53* in mice is not

sufficient to overcome this barrier, suggesting that oncogene-induced senescence in this context depends on more complex regulatory networks [29]. However, when caerulein-induced CP is combined with the loss of $p16^{Ink4a}/p19^{Arf}$, the mice show similar numbers of low-grade PanINs, indicating similar sensitivity to tumor-initiating insults, but the number of high-grade lesions is significantly increased and the overall survival of the mice is shorter. This observation was interpreted as loss of the barrier to progression from low-grade to high-grade PanINs. Consistently, the expression of senescence markers is lost in low-grade PanINs in the mice treated with caerulein [29]. Most importantly, senescence markers are detected in low-grade PanINs in patients with untreated CP but not in those from patients with CP treated with anti-inflammatory drugs, nor in PDAC [29, 103].

Recently, extensive massive parallel sequencing and bioinformatics efforts have provided convincing evidence that the progression from low-grade to high-grade PanINs and then to PDAC might not necessarily be as linear as it was originally hypothesized, and that PanINs might not be the precursors of PDAC [104]. This new concept of PDAC progression, however, does not exclude that the actual precursor cells might undergo a senescence-like process, such as PanINs, which is also inhibited by pancreatitis.

Contribution of the Nonepithelial Compartment: A Cellular Orchestra

Although epithelial cells constitute the vast majority of the normal pancreatic parenchyma, other cell types are present and play a major role in tissue homeostasis and during response to injury, including pancreatitis (Fig. 2). Fibrosis and stromal activation are characteristics of both CP and PDAC, and the histological composition of the stroma is similar in both pathologies, with the presence of activated α-smooth muscle actin (α-SMA)-positive PSC, macrophages, and many other inflammatory cells. The well-orchestrated continuous exchange of signals between epithelial and stromal cells, as well as among distinct populations of stromal cells, is essential for tissue regeneration after pancreatitis, and is hacked by oncogenes such as *KRas* to generate the protumorigenic and immunosuppressive environment that is almost universally observed in PDAC.

The role of microenvironment in PDAC initiation and progression will be extensively explained in another chapter of this book, therefore the available data supporting the importance of PSC and inflammatory cells during pancreatitis will only be mentioned.

Pancreatic Stellate Cells

PSC are present in the normal pancreas, mainly with a periacinar distribution, and they are in a quiescent state, characterized by the presence of vitamin A-containing lipid droplets. Their function in the normal pancreas is still not fully established. In response to pancreatic injury and inflammation, PSC are rapidly activated, express α-SMA characteristic of myofibroblasts – which are proliferative and migratory –

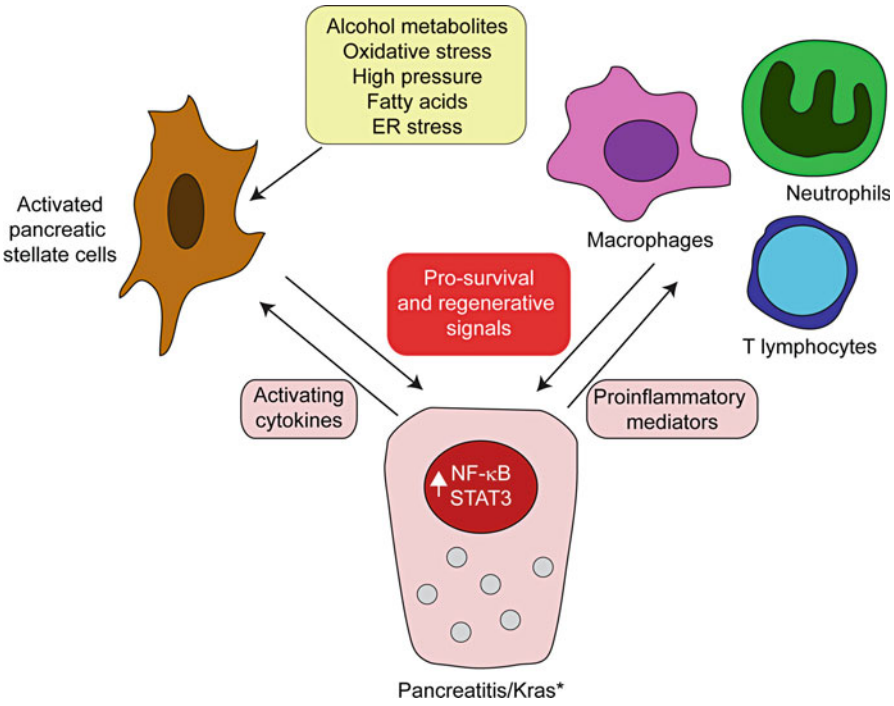

Fig. 2 Cellular mechanisms involved. In the normal pancreas, >95% of cells are epithelial. However, in pancreatitis and PDAC there is a dramatic increase in the number of stromal and haematopoietic cells that cross-talk with exocrine epithelial cells activating multiple signaling pathways that contribute to modulate their functional status. Depicted are the major players contributing to this cross-talk

and produce abundant ECM proteins, as well as matrix metalloproteases and their inhibitors, indicating a pivotal role in ECM homeostasis [105].

Activation of PSC during AP is important for the successful regeneration of the tissue, mainly because of the mentioned role in ECM production and degradation. In particular, the equilibrium between ECM production and its degradation is essential for the full recovery and the fact that no fibrosis is observed in AP strongly indicates that this equilibrium is not perturbed by mild injury. On the contrary, upon prolonged injury, such as in CP or PDAC, the equilibrium is disrupted towards the excessive ECM deposition that finally produces the characteristic fibrotic microenvironment observed in both cases [106].

In vitro experiments have shown that PSC can be activated by multiple factors that are involved in pancreatitis, including alcohol and its metabolites, oxidative stress, pressure, growth factors such as platelet-derived growth factor and TGF-β, and cytokines produced by the damaged acinar cells or by resident and recruited inflammatory cells, such as Il-1, Il-6, and tumor necrosis factor α (TNF-α) [106]. In turn, PSC also produce cytokines and growth factors that contribute to maintain their

own activation, in a paracrine loop, as well as chemokines such as Il-8 and monocyte chemoattractant protein 1, which contribute to the recruitment of inflammatory cells [107]. PSC also produce toll-like receptors (TLRs), which activate the recruited immune cells [108]. Importantly, modulation of the ECM can also restore PSC to quiescence, providing opportunities for pharmacological intervention [109, 110]. In conclusion, the current understanding of the role of PSC in fibrosis and cancer is that they initially function to sustain the immune-mediated resolution of the damage and the regeneration of the tissue; however, when the damage is prolonged, such as in CP or in the presence of mutant KRas, the equilibrium of their multiple activities is perturbed in favor of the generation of a fibrotic and immuno-suppressive environment, which favors PDAC development and progression.

Inflammatory Cells

Animal models of AP have demonstrated that, in response to the initial insult, acinar cells produce and release multiple inflammatory mediators, including Il-1β, Il-6, and Tnf-α, that first recruit neutrophils and then macrophages, monocytes, and lymphocytes. Activation of the transcription factor NF-κB is known to be crucial in the induction of the cytokine production by acinar cells, and its role will be discussed in more detail below [111]. Once inflammatory cells are recruited to the pancreas, the inflammasome is activated in macrophages and contributes to the severity of pancreatitis. The inflammasome is induced also by damage-associated molecular pattern molecules, which are released by damaged or dying cells [112]. In animal models of AP, as in most patients with AP, the inflammatory phase resolves and tissue homeostasis is recovered.

Compared to AP, much less is known about how inflammation is first induced in CP. Patients with CP show pancreatic infiltration of macrophages, T- and B- lymphocytes, and in particular immunosuppressive Tregs [113]. Similarly, both macrophages and T-lymphocytes have been observed in animal models of CP, which are normally based on repetitive induction of AP, as explained above [114]. It is possible that the persistent inflammation observed in CP is also initiated by signals released by damaged acinar cells, which induce the recruitment of inflammatory cells and the activation of the inflammasome, as in AP, although the exact mechanisms that impede the resolution of inflammation are not fully clear.

It is known that up to 50% of the whole tumor cell mass in PDAC can be composed of stroma including ECM, mesenchymal cells, and immune cells, including macrophages, myeloid-derived suppressor cells, neutrophils, dendritic cells, and B- and T-lymphocytes [115]. This cell composition is rather similar to what observed in CP, suggesting that pancreatitis might provide a favorable landscape for the proliferation of precancerous cells and their progression to carcinoma. Importantly, persistent autocrine and paracrine loops mediated by interleukins – such as Il-6, TNF-α, and Il-1α – are present between myeloid cells and epithelial cells in the preneoplastic and neoplastic inflammatory environment, which maintain and amplify the activation of pro-proliferative and proinflammatory signals such as the Stat3 and NF-κB pathways [116, 117].

Signaling Pathways Relevant to the Pancreatitis-to-Cancer Sequence

The signaling mechanisms that are involved in triggering the changes in epithelial differentiation described above emanate from both the causal events of pancreatitis and the cross-talk of epithelial cells with mesenchymal and haematopoietic cells (Fig. 2). The focus here will be on the signaling pathways that have been shown to contribute to the pancreatitis-to-cancer sequence (Fig. 3). They are particularly relevant because these biochemical mechanisms may be more amenable to pharmacological targeting than the transcription factors involved in developmental/cellular reprogramming described above.

MAP Kinase

While it is textbook knowledge that mutant KRas activates the MAP kinase pathway constitutively, there is scarce evidence of downstream signaling – and even less of cellular changes – when the oncogene is activated in pancreatic precursors or in adult

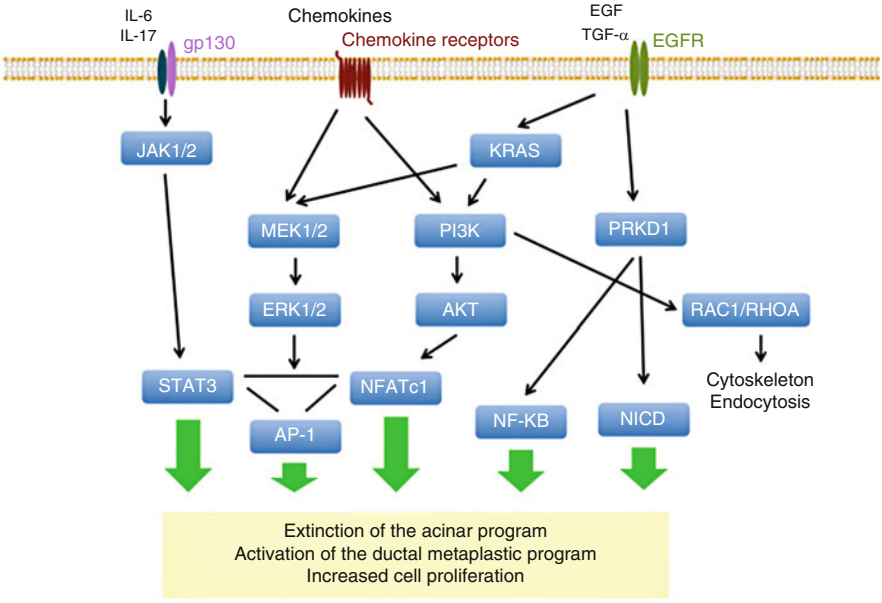

Fig. 3 Signaling pathways involved in the regulation of cellular phenotypes that favor the pancreatitis-to-pancreatic cancer progression. KRAS activation status is modulated through both genetic (mutation) and nongenetic (biochemical regulation from receptor tyrosine kinases, among others) mechanisms and plays a crucial role in pancreatic cancer development. Cytokine receptors, through the JAK/STAT pathway, and chemokine receptors, through the MAPK and PI3K pathways, contribute to the deregulation of cell fate. Signals contributing to this process emanate from both epithelial and nonepithelial cell types, as outlined in Fig. 3

acinar cells [64, 118]. In vitro studies have shown that basal levels of active KRas in acinar cells from KC pancreata are low but they can be markedly increased by adding epidermal growth factor (EGF), suggesting that there is ample opportunity to modulate RAS activity even in cells with mutant *KRas* [119]. Using a doxycycline-inducible mutant *KRas* (iKRas*) model, Pasca di Magliano and colleagues have shown that sustained expression of mutant KRas is required for PanIN formation, both spontaneously and as a result of pancreatitis induction [120]. Acute pancreatitis leads to sustained MEK/MAPK activation in the presence of mutant KRas, but not in WT mice, and the administration of the MEK1/2 inhibitor PD325901 not only leads to reduced development of ADM and PanINs but also to a lower number of established PanINs. This effect is mediated by an increase in cell death and the redifferentiation of cells to acquire an acinar phenotype, through the upregulation of key pancreatic transcription factors such as Mist1 and the participation of epithelial and stromal/haematopoietic cross-talk [120, 121].

The contribution of MEK to the tumor-promoting effect of caerulein pancreatitis could be mediated, at least in part, by the regulation of ADM. Pharmacological inhibition of MEK1/2 with Trametinib, and genetic deletion of *Mek* in epithelial cells, reduces ADM in vitro and in vivo but it does not affect AP. In established caerulein CP, systemic and epithelial MEK inhibition reduces acinar loss and fibrosis but systemic MEK inhibition impairs epithelial cell proliferation and pancreas regeneration, indicating that MEK activity in both epithelial and nonepithelial cells contributes to chronic pancreatic damage [122] that may favor tumor progression.

Different arms of the MAP kinase pathway can contribute differently to the pancreatitis-to-PDAC progression. Accordingly, MKK4 and MKK7 are required for acinar redifferentiation upon damage, possibly through sustained expression of Sox9 and other transcriptional regulators, and have been shown to act as tumor suppressors in the KC model [123].

The RAS-MAPK, EGF receptor (EGFR), and NF-kB pathways, as well as endocytic traffic and Golgi integrity, are also regulated by the Prkd1 kinase, which is required for EGFR ligand- and mutant KRas-driven ADM in vitro and in vivo and is sufficient to induce metaplasia in vitro [124].

Epidermal Growth Factor Receptor

EGFR signaling is required for regeneration upon induction of pancreatitis, in part through Agr2, which contributes to the membrane expression of EGFR and downstream signaling [125]. A role of EGFR and its ligands in ADM was first shown by transgenic overexpression of TGF-α in the pancreas [126] and subsequently in vitro through the activation of Notch [127]. More recently, it has been shown that EGFR is activated during mutant KRas-driven ADM and PanIN formation, and pharmacological and genetic inhibition of EGFR suppresses PDAC initiation through the regulation of MAPK activation. These studies suggest that EGFR contributes to reach the threshold of active mutant KRas required for ADM during tumor initiation. The effects of EGFR are mediated by ADAM17, a mechanism that also participates

in pancreatitis-stimulated PDAC development [29, 128]. The requirement of EGFR for PDAC initiation is independent of senescence induced by mutant KRas, as shown by the concomitant inactivation of $p16^{Ink4a}/p19^{Arf}$, but it is completely overridden by the inactivation of *Trp53* in pancreatic epithelial cells [29]. In the mutant KRas model, EGFR can also activate Nfatc1 which – in cooperation with AP1 – upregulates Sox9 expression [129] that is critically required for ADM and PDAC initiation [130].

PI3K Pathway

The PI3K pathway is activated downstream of mutant KRAS. In KC mice, genetic inactivation of p110α in epithelial cells using a kinase-dead mutant, as well as pharmacological inhibition, reduces AKT activation and caerulein-mediated damage, and PanIN and PDAC formation [131, 132]. By contrast, inhibition of p110β has no effects. p110α has been shown to induce cytoskeletal reorganization through the regulation of small GTPases and there is strong evidence that activation of Rac1 in the KC model is required for ADM, PanIN formation and PDAC progression, downstream of p110 activation [131–133], in part through the regulation of endocytosis [134].

Stat3

The STAT transcription factors are regulated by phosphorylation by the Janus-activated kinases (JAK) that, in turn, are downstream of the gp130 coreceptor involved primarily in cytokine signaling. This pathway crucially links inflammation and carcinogenesis in multiple tissues, including the pancreas. Pap1 (Pancreatitis-associated protein) was shown to suppress the inflammatory response during pancreatitis and – in vitro – it inhibits the NF-κB pathway in a Stat3-dependent manner [135]. In vivo, genetic deletion of *Stat3* in pancreatic cells suppresses ADM resulting from transgenic overexpression of Pdx1 [136] and pharmacological inhibition of JAK with AG490 ameliorates caerulein AP in rats [137]. In the KC model, Fukuda et al. showed that mutant KRas activates Stat3 and that deletion of *Stat3* in pancreatic cells suppresses spontaneous and caerulein-induced inflammatory cell infiltration and cytokine mRNA production, ADM, and PanIN formation [86]. The activation of Stat3 in epithelial cells was subsequently shown to be primarily caused by Il-6 trans-signaling, a mechanism that involves myeloid Il-6 secretion and binding to the soluble Il-6 receptor which then engages gp130 and JAK/STAT signaling in acinar cells leading to the secretion of Cxcl1 [116]. While this mechanism was initially shown to play a role in systemic effects of AP, namely lung injury, subsequent work from the same group revealed that Il-6 trans-signaling and Stat3 activation upregulates survival (Mcl-1, Bcl-x, and survivin) and cell cycle pathways (cyclin D1 and c-myc) and is required for the progression of PanIN to PDAC in the KC mouse model. The crucial role of Stat3 was additionally revealed by genetic deletion

of the suppressor of cytokine signaling *Socs3* in epithelial cells, which accelerates PanIN to PDAC progression [116]. Reg3β, a pancreatitis-response factor, is regulated in response to Il-17 and – in turn – it activates the JAK/Stat3 pathway; its systemic deletion in KC mice results in reduced PanIN formation and PDAC initiation [138]. A link between Stat3 activation and the transcriptional control of pancreatic homeostasis has been also established using mice heterozygous for *Nr5a2*, a crucial regulator of acinar differentiation [88], which display increased inflammation and ADM in response to induction of pancreatitis, in association with Stat3 induction in acinar cells.

Stat3 also cooperates with – and binds to – Nfatc1, which is activated by inflammation and itself promotes inflammation-driven carcinogenesis in KC mutant mice. Chromatin immunoprecipitation, followed by massive parallel sequencing, has shown that Stat3 is required for the binding of Nfatc1 at enhancers to regulate the expression of cancer genes such as EGFR and cyclin D3, and that genetic and pharmacological inhibition of Nfatc1 attenuates the protumoral activity of Stat3 [139]. In *Ela-CreERT2* mice in which PDAC is induced by oncogene activation in adult acinar cells followed by pancreatitis, YAP1 and TAZ – two major transcriptional regulators of the Hippo pathway which are upregulated upon caerulein-induced pancreatitis – were shown by genetic means to be required for the upregulation of multiple components of the JAK/Stat3 pathway [140].

NF-KB Pathway, Autophagy, and COX2

Several studies have consistently shown that suppressed activity of the NF-κB pathway is critical for pancreatic homeostasis. In adult acinar cells, inducible overexpression of the p65 subunit does not have a major phenotype, likely due to compensation by an increased expression of the Ikkα inhibitor. By contrast, prolonged induced transgenic overexpression of Ikk2 leads to increased NF-κB activity and pancreatitis, associated with loss of acinar cells, PSC activation, and fibrosis [52]. Accordingly, *Ikk2* deletion prevents the development of preneoplastic lesions [141]. In agreement with these findings, deletion of *Ikkα* in the pancreas causes acinar loss and a spontaneous pancreatitis phenotype. In *Trp53* WT mice, persistent NF-κB activation leads to CP, but no tumors, likely through regulation of DNA repair and/or apoptosis. However, upon *Trp53* inactivation, Ikk2-overexpressing mice show hyperactivation of the MAPK and Hippo pathways and increased Myc activity and develop a wide variety of *KRas*-wild type tumors [55].

The pancreatic changes observed in Ikk2-overexpressing mice are associated with an accumulation and aggregation of the autophagy regulator p62 and with increased ER and oxidative stress. The deletion of *p62* attenuates all of these processes, indicating that the role of NF-κB in pancreatic homeostasis is due – at least in part – to its cross-talk with autophagy pathways [68]. This notion is further supported by the fact that inactivation of *Atg5* [69] or *Atg7* [70] in pancreatic epithelial cells leads to severe acinar degeneration and a chronic pancreatitis-like phenotype with increased epithelial cell proliferation, inflammation, and fibrosis. ER stress is

evidenced by increased Perk and eIF2a phosphorylation and Chop levels and by partial loss of the rough ER with a reduction in protein synthesis. *p62* deletion attenuates these changes but ER stress, reduced protein synthesis, oxidative and DNA damage persist. The antioxidant butylated hydroxyanisole also partially restores the acinar phenotype with increased amylase expression and reduced fibrosis but no attenuation of inflammation, ER stress, p62 accumulation, or mTORC inhibition [70].

COX2 is another mediator of the effects of NF-κB activation. In mice expressing mutant KRas in the pancreas, inflammatory stimuli trigger NF-κB activation and Cox2 expression, leading to persistent KRas activation, downstream signaling, and PanIN formation. Deletion of *Cox2* has similar effects as deletion of *Ikk2*, suppressing the development of preneoplastic lesions [141]. Cox2 is induced in epithelial and nonepithelial cells during pancreatitis and its sole transgenic overexpression in adult acinar cells is sufficient to cause acinar atrophy, stellate cell activation, matrix deposition, inflammatory infiltrates, epithelial cell proliferation and DNA damage; after 20 weeks, a fraction of mice develops PanIN-1 (30%) and PanIN-2 (5%) lesions without PDAC. However, sustained Cox2 overexpression in a p53-null context leads to increased MAPK activity and development of KRas-wild type tumors [55].

A role in progression from CP to PDAC has been shown in the model of Guerra and Barbacid, where Sulindac – a COX1/2 inhibitor – reduces the number of low-grade, high-grade PanINs, and PDAC [29]. In the KC model, Cox2 is required for the high fat diet-induced inflammation, fibrosis, and KRas activation that results in enhanced PanIN and PDAC development [142].

Conclusions and Implications for Preventive/Therapeutic Opportunities

Epidemiological, clinical, and experimental evidences support the notion that pancreatic inflammation plays an important role in malignant transformation in the pancreas. The animal models and in vitro studies have provided insightful evidences regarding the mechanisms involved in the pancreatitis-to-cancer progression.

The work described above, largely based on the use of genetic mouse models of pancreatitis-to-PDAC progression, provides the basis for a variety of preventive/therapeutic opportunities. Three important aspects for the translation of this knowledge will be:

1. The selection of patients for clinical trials. Except for patients with hereditary chronic pancreatitis – who have a huge increase in PDAC risk but are rare – all other patients with CP have a lower risk.
2. The identification of intermediate biomarkers of response. Because the natural history of progression from CP to PDAC is long, studies would benefit from the use of surrogate markers of disease progression including inflammatory biomarkers or – possibly – detection of somatic genetic alterations in liquid biopsies.

3. The selection of drug interventions. Approved drugs such as COX2 inhibitors or vitamin D may be easier to test through investigator-initiated trials. By contrast, it may prove more challenging to justify trials testing the effects of chronic treatment with the inhibitors of EGFR, MEK1/2, JAK/STAT and other related signaling pathways, particularly in low/intermediate risk populations.

Cross-References

- ▶ EGFR (ErbB) Signaling Pathways in Pancreatic Cancer Pathogenesis
- ▶ Epidemiology and Prospects for Prevention of Pancreatic Cancer
- ▶ Developmental Molecular Biology of the Pancreas
- ▶ Mouse Models of Pancreatic Exocrine Cancer
- ▶ Notch Signaling in Pancreatic Morphogenesis and Pancreatic Cancer Pathogenesis
- ▶ Stromal Inflammation in Pancreatic Cancer: Mechanisms and Translational Applications
- ▶ The Molecular Pathology of Precursor Lesions of Pancreatic Cancer

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Pancreatic Cancer Stem Cells

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Abstract

Cancer stem cells (CSCs) are a distinct subpopulation of cells within a tumor that are capable of self-renewal and producing differentiated progeny. These cells appear to be more resilient to treatment than bulk tumor cells. Pancreatic CSCs have distinct markers; the most common identifiers are CD44, CD24, ESA, and CD133; however, other surface markers, characteristics, and intracellular signaling have been found to be unique to this population of tumor cells. New studies also indicate that CSCs may also have a distinct metabolic profile that distinguishes them from non-CSC tumor cells. There are many promising new targets on the horizon to strategize how to inhibit the growth of pancreatic CSCs by capitalizing on these features. However, many questions must be answered in order to translate this knowledge into therapeutic treatments for patients.

Keywords

Cancer stem cells · Pancreatic cancer · CD44 · CD24 · ESA

Introduction

One of the great barriers to treating pancreatic cancer is the relative resistance to standard treatments, including radiation and chemotherapy. Surgical resection remains the only potential curative treatment; however, only 15–20% of patients have disease amendable to surgical resection at the time of diagnosis. Despite surgery, nearly 85% of patients will die of their disease due to undetected micrometastasis at the time of treatment [1, 2]. Emerging studies show that underlying this resistance is a distinct population of cancer cells termed cancer stem cells (CSCs). Cancer stem cells are thought to comprise a very small portion of pancreatic tumors, in many cases as few as 0.2–5% of the cancer cell population. Intriguingly, this small population of cells has been implicated in carcinogenesis, early metastasis, and drug resistance in multiple solid tumor types, including pancreatic cancer [3–6]. In patients where the bulk tumor appears to be eradicated by therapy and relapse occurs, there is increasing experimental evidence that small populations of CSCs have not been destroyed and are responsible for disease recurrence [7–11] (Fig. 1). Rapidly expanding evidence in the field of CSCs makes them an attractive target for future therapeutic strategies.

The general definition of a CSC parallels that of nonmalignant stem cell: a cell that is capable of both self-renewal and propagation of differentiated progeny [12]. Currently, there is no precise consensus on the definition of a CSC as multiple cell surface markers and transcriptomic and genomic "signatures" of cancer cell populations possessing stem cell-like features have been identified. This has prompted the use of the term tumor-initiating cells (TIC) or stem-like cells interchangeably with the term CSCs [13–15]. Regardless of terminology, there is a large amount of evidence that these populations play a paramount role in tumorigenesis and therapeutic evasion that warrants ongoing investigation.

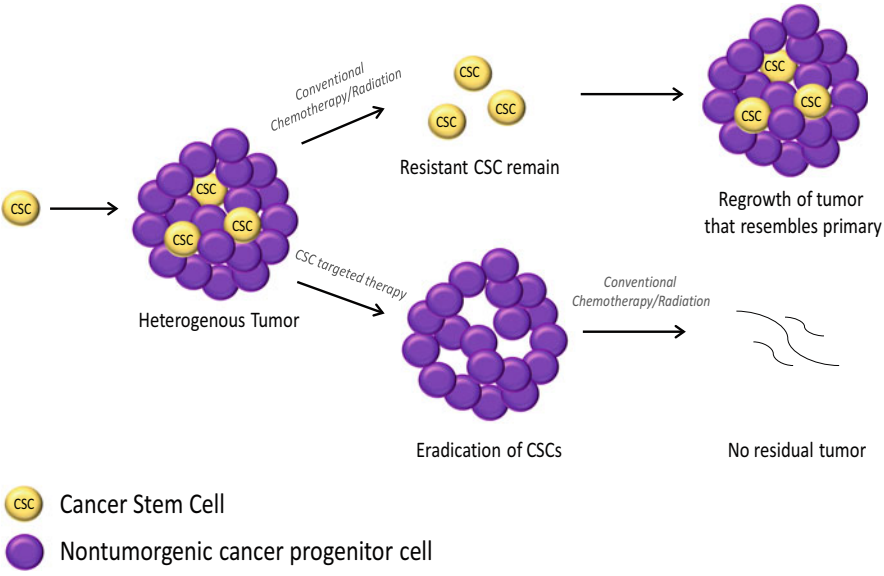

Fig. 1 The cancer stem cell theory. Conventional therapies such as chemotherapy and radiation only eradicate bulk tumor cells and leave CSC behind to regenerate the primary tumor. Future therapeutic targets that can eradicate CSCs can be used in combination with conventional therapies to eliminate all components of a tumor

The cancer stem cell hypothesis was put forth over 150 years ago [16]; however, proof of the existence of CSCs came many years later in 1997 when John Dick and colleagues first identified cancer stem cells in acute myeloid leukemia [17]. This discovery was enhanced by advances in techniques such as fluorescence-activated cell sorting (FACS) analysis which allowed more efficient separation of subpopulations of cells based on rare cell surface markers in combination with in vivo limiting dilution assays in immunodeficient mice. This pioneering work in leukemia demonstrated that these rare subpopulations ($CD34^+/CD38^-$) could regenerate a tumor identical to the parent neoplasm with very few cells. In contrast, tens of thousands of leukemic cancer cells lacking this phenotype were not tumorigenic. In 2003 Al-Hajj et al. opened up the field of cancer biology by identifying CSC in solid tumors, where they noted a subpopulation of CSCs in human breast cancer specimens [18]. They found that as few as 100 $CD44^+/CD24^-$ cells could initiate tumor formation and that these cells could undergo self-renewal and also produce more differentiated cell populations in secondary recipient mice. Subsequently, CSCs have been identified in multiple solid tumors including pancreatic cancer by Li et al. [3] and Hermann et al. in 2007 [4]. Li et al. studied human primary pancreatic tumors and low-passage primary tumors expanded as xenografts to identify a population of putative pancreatic cancer stem cells with the surface markers $CD44^+$ $CD24^+$ ESA^+. The pancreatic cancer stem cells demonstrated a 100-fold greater tumor-initiating potential than tumor cells that were negative for these markers. Data generated from ten tumors showed that cells expressing the three surface markers, $CD44^+$ $CD24^+$ ESA^+, comprised only 0.2–0.8% of all human pancreatic

cancer cells. This rare population had the highest tumorigenic potential when injected into NOD-SCID mice, with only 100 cells with stem cells markers $CD44^+$ $CD24^+$ ESA^+ being required to form tumors in 6 out of 12 mice. Subpopulations of tumor cells that were negative for cancer stem cells markers $CD44^-$ $CD24^-$ ESA^- were much less tumorigenic, with 10,000 cells required to generate tumors in 1 of 12 mice.

Hermann et al. identified a population of CSCs in human pancreatic tumor specimens using the hematopoietic stem cell marker CD133+ [4]. Flow cytometry analysis demonstrated that bulk tumors contained 1–3.2% CD133+ cells. Orthotopic implantation of as few as 500 CD133+ cells was able to generate tumors in athymic mice that reproduced the primary tumor at the histological level, whereas 10^6 CD133− cells were not found to be tumorigenic. This population of CSC did not express the epithelial differentiation marker cytokeratin, suggesting that they are a progenitor population distinct from the bulk tumor cells. Co-expression of CD44 and CD24 was observed in ∼0.1% of $CD133^+$ CSCs, suggesting the possibility that multiple, distinct populations may exist. Furthermore, this population of pancreatic CSCs was enriched in xenograft tumors following gemcitabine treatment, implicating this population in therapeutic resistance.

Characterization of Pancreatic CSCs

It is agreed that CSCs possess the ability to self-renew and create differentiated progeny. In vivo criteria to define CSCs come from limiting dilution assays in immunodeficient mice which demonstrate the ability of CSCs to regenerate malignant populations that histologically resemble the parental cancer. Hallmarks of CSCs in vitro include slow proliferation and the ability to form spheres in nonadherent cultures due to their anchorage-independent growth properties [19]. There is currently no consensus on the phenotypic or genotypic characterization of pancreatic CSCs. Multiple studies have identified rare populations of cells in pancreatic cancer primary cultures or cell lines that meet the above criteria; however, no single marker is common to each study. Furthermore, the biological function of the respective surface markers and their contribution to "stemness" of the expressing cell remains unclear in most cases [6]. This indicates that there may be an array of phenotypes of pancreatic CSCs that may be influenced by the specific microenvironment, that CSCs with varying surface markers may represent various stages of differentiation, or that there is a common, currently unidentified, marker uniting these populations (Table 1).

CD44, CD24, and ESA

Li et al. demonstrated that CD44+ CD24+ ESA+ cells isolated from xenograft tumors originating from patients with pancreatic cancer showed a 100-fold enrichment for tumor formation compared to CD44− CD24− ESA− cells [3]. These markers were chosen based on previous work demonstrating breast CSCs had

Table 1 Pancreatic cancer stem cell markers

Marker	First author, year
CD44+/CD24+/ESA+	Li, 2007 [3]
CD44+/c-Met^{high}	Li, 2011 [31]
CD133+	Hermann, 2007 [4]
CD133+/CXCR4+	Hermann, 2007 [4]
ALDH1	Kim, 2011 [13]
Autofluorescence	Miranda-Lorenzo, 2014 [42]
Side populations (ability to efflux the Hoechst 33342 dye)	Neiss, 2015 [6]

similar properties [18]. In vitro work in Panc-1 cells demonstrated that CD44+ CD24 + cells comprise 2.1–3.5% of the culture population and that these cells have a 20-fold increase in tumorigenicity compared to cells negative for these markers [20]. It is unclear if these unique cell surface markers functionally contribute to the pancreatic CSC phenotype or if their expression is a by-product of upstream signaling events in pancreatic CSCs. CD24 is a heavily glycosylated cell surface protein that plays an important role in cell selection and maturation during hematopoiesis. CD24 is also known to be an alternative ligand for P-selectin and may facilitate cell-cell interactions and has been proposed to play a role in metastasis. Interestingly, growth of multiple pancreatic cancer cell lines has been shown to be dependent on CD24 signaling [19, 21]. ESA (also known as EpCAM) is expressed on the basolateral cell surface of most human simple epithelia and is also expressed in the vast majority of carcinomas lending to its common use as a tumor marker [22]. CD44 is a cell surface glycoprotein that is broadly expressed by cells of epithelial, mesenchymal, and hematopoietic origin. It is involved in cell-matrix adhesion, survival, and growth and has been implicated to have a role in tumorigenesis and metastasis [23]. CD44 is thought to have a functional role in CSC biology in regulating stemness, as a splice variant of CD44 has been shown to activate the ectodomain of c-Met. CD44-positive cells have been identified as a population of cells that leave the pancreas early to disseminate systemically in a Cre-lox-based mouse model of PDA that was used to study the fate of pancreatic epithelial cells during various stages of tumor progression. In this model, Rhim et al. used a YFP lineage label to identify PDA cells that had completed an EMT and then examined the proportion of YFP+ cells in the circulation expressing CD44+/CD24+. Using fluorescence-activated cell sorting (FACS) analysis, it was shown that 23.1% ± 12.9% and 46.4% ± 14.7% of sorted YFP^{+} circulating pancreatic cells from PanIN and PDA samples were found to be CD24^{+}CD44^{+}, representing a greater than 100-fold enrichment when compared to the source pancreas [24]. Furthermore, work by Wang et al. identified in a novel PDA mouse model expressing ATDC (ataxia-telangiectasia group D complementing gene) that, in the presence of oncogenic KRAS, the formation and the development of invasive and metastatic cancers were markedly accelerated. It was shown that ATDC upregulates CD44 in mouse and human PanIN lesions via activation of β-catenin signaling, leading to the induction of a CSC/EMT phenotype [25]. Knockdown of CD44 in primary colon

cancer cell lines reduces clonogenicity in vitro and tumorigenicity in vivo [8]. Interestingly, CD44 is also a receptor for the glycosaminoglycan (HA). HA is found in high levels in the extracellular matrix in PDA. HA signaling via CD44 and other receptors has been found to regulate receptor tyrosine kinase and small GTPase activity and is implicated in the processes of angiogenesis, epithelial-mesenchymal transition, and chemoresistance [26]. This evidence suggests that the stroma may contribute to the ongoing survival of PCSCs and that current clinical trials using recombinant human hyaluronidase in the treatment of PDA may be effective, at least in part, by affecting pancreatic CSC function [27].

CD133

CD133 is a transmembrane glycoprotein expressed on normal stem cells and progenitor cells. CD133 has also been shown to identify CSC populations in multiple solid tumors, including pancreatic cancer [4, 7, 8, 28]. Hermann et al. demonstrated that CD133+ cells had increased tumorigenicity compared with CD133− cells and that the CD133+ population was enriched in xenograft tumors in mice treated with gemcitabine. Interestingly, they reported partial overlap of CD133+ cell populations with CD44+ CD24+ ESA+ population. Further studies comparing CD44+ CD24+ ESA+ cells and with CD44+ CD24+ ESA+ CD133+ cells may elucidate the functional role of this protein and further examine its role in drug resistance.

CXCR4

CXCR4 is a chemokine receptor that has been found to play a role in invasion and metastasis, as it was found to be elevated at the invasive edge of pancreatic tumors. In isolation, CXCR4 is not a marker of CSCs per se; however, it was shown to mark a subpopulation of $CD133^+$ pancreatic CSCs with a high propensity to metastasize [4]. CXCR4 is the receptor for stromal-derived factor-1 (SDF-1/CXCL12) and is important for hematopoietic stem cell homing to the bone marrow and metastasis and proliferation of cancer cells [29, 30]. Importantly, blocking $CD133^+/CXCR4^+$ cells prevented metastasis of tumor xenografts in mice. These data indicate that CXCR4 might serve as target for therapeutics designed to slow or prevent metastasis of pancreatic CSCs. Like $CD133^+/CXCR4^-$ cells, $CD133^+/CXCR4^+$ cells were resistant to cell death induced by gemcitabine, indicating the need for new approaches to effectively eliminate this cell population and prevent cancer relapse.

c-MET

The mesenchymal-epithelial transition factor gene *c-MET* is a membrane-bound receptor tyrosine kinase that has previously been identified on normal pancreatic stem and progenitor cells [31]. c-MET overexpression is associated with a stem

cell-like phenotype in a wide range of cancers, and the interaction of c-MET with its ligand hepatocyte growth factor (HGF) (also referred to as scatter factor) has been shown to promote malignancy and tumor drug resistance [32]. In the mutated or amplified form, c-MET generates and maintains the transformed phenotype and drives clonal evolution of tumorigenesis; however, the wild-type form of c-MET seems to contribute to the maintenance of the CSC phenotype [9, 33]. Li et al. examined pancreatic CSCs for the presence of c-MET and compared these subpopulations with other known pancreatic CSC markers such as $CD44^+/CD24^+/ESA^+$ and CD133+. Work in primary human pancreatic cancer cell populations demonstrated that cells expressing high levels of c-MET were as tumorigenic as $CD44^+/CD24^+/ESA^+$ cells and more tumorigenic than $CD133^+$ cells. Interestingly, $CD44^+/c\text{-}MET^{High}$ cells were the most tumorigenic of all populations, whereas $CD133^+/c\text{-}MET^{High}$ cells were comparatively less tumorigenic in vivo. Tumors formed in mice from $CD44^+/c\text{-}MET^{High}$ cells were identical to the original tumors from which they were derived. In addition, cells that express CD44+ and c-MET together were found to be more tumorigenic in mice than cells that express c-Met alone. Expression of c-MET also correlated with the ability of cells to form tumorspheres in vitro [19, 31]. It is possible that CD44 and c-Met may work in concert to promote a pancreatic CSC phenotype as CD44 is important for optimal HGF signaling via c-MET.

There is a unique appeal to c-MET as a pancreatic CSC target, as there are specific inhibitors of c-MET, unlike many other pancreatic CSC markers. Findings indicate that agents that disrupt c-MET activity might interfere with CSC activities in different tumor types, and experimental evidence suggested that c-MET plays a dual role in oncogenesis. Inhibition of c-MET activity with the kinase inhibitor XL184 (cabozantinib) reduced tumorsphere formation, growth of tumor xenografts, and metastasis in intracardiac injection models. XL184 also increased the efficacy of gemcitabine against subcutaneous and orthotopic xenograft tumors, further demonstrating its potential clinical utility [31]. The role of c-MET in CSC function was highlighted in a study showing that high expression of c-MET in glioblastoma cells correlated with increased formation of neurospheres in vitro, tumorigenesis in vivo, resistance to radiation, and expression of stem cell transcription factors, such as Nanog and SOX2 [34–36].

ALDH1

Aldehyde dehydrogenase 1 (ALDH1) has been shown to enrich for normal and malignant stem cell populations in multiple organ systems [37, 38]. Unlike the previous phenotypic markers discussed, ALDH1 is not found on the cell membrane but as it is an intracellular enzyme involved in retinoic acid metabolism. This distinct marker was first studied in CSC populations in hematological malignancies. Hess et al. demonstrated hematopoietic stem cell populations expressing that $ALDH^{high}$ and CD133+ were able to reconstitute the bone marrow with a tenfold greater capability compared to cells enriched for CD133+ expression alone [39]. Rasheed et al. demonstrated increased tumorigenic potential of

$ALDH^{high}/CD44^{+}/CD24^{+}$ and $CD44^{+}/CD24^{+}$ pancreatic cancer cell populations with ALDH expression correlating with a worse prognosis in early-stage pancreatic cancer patients [40]. Pancreatic cancer xenograft tumors exposed to gemcitabine become enriched for ALDH1+- and CD24+-positive cells, indicating that they can withstand chemotherapy [41]. Interestingly, these authors found minimal overlap between ALDH and $CD44^{+}/CD24^{+}$ cell populations (<0.1%), suggesting the existence of at least two distinct tumor-initiating populations within human pancreatic tumors.

Autofluorescence

In 2014, the unique CSC property of autofluorescence was discovered by Miranda-Lorenzo et al. in human pancreatic tumors [42]. This significant finding allowed identification of CSC independent of cell surface markers. The interest in this CSC biomarker stems from the fact that expression of cell surface markers is subject to change due to different tissue digestion protocols and isolation techniques. This alternate detection method is based on cellular autofluorescence following exposure to a standard blue laser which is thought to be a result of the accumulation of the fluorescent vitamin riboflavin in ABCG2-coated vesicles exclusively located within the cytoplasm of CSCs. Cells that possess this property demonstrated CSC features and phenotypes, such as self-renewal, exclusive long-term tumorigenicity, and invasiveness in vivo. This population was found to compose 0.04–6.38% of bulk pancreatic tumor cells. Autofluorescence allowed this rare population to be detected by flow cytometry and avoided some of the possible artifacts of surface markers. It was found that the CSC surface markers CD44, CD133, and CXCR4 were variably overexpressed in autofluorescent cells; however, none of these markers was exclusively restricted to autofluorescent cells, suggesting that these may represent a discrete subpopulation within the CSCs.

Other CSC Subpopulations

Work by Niess et al. in 2015 identified another potential measure of pancreatic CSCs that is independent of cell surface markers. The unique phenotype of these CSC cells is thought to partially depend on the ABCG2 transporter, which bears a similar mechanism to autofluorescence CSC populations. Putative CSCs referred to as "side populations" (SP) were isolated from bulk tumor cells based on their ability to efflux the Hoechst 33342 dye. Cells that are able to efflux Hoechst dye are thought to harbor cell membrane transporters, such as the ABCG2 transporter, giving cells the ability efflux chemotherapeutics which is thought to augment therapeutic resistance. SP cells have been previously identified in multiple CSC populations including hepatocellular carcinoma [43], melanoma [14], glioma [44], and esophageal and lung cancer [10, 15]. In this study, SP cells were isolated from the human pancreatic cancer cell line L3.6pl, and tumorigenicity was evaluated following orthotopic

injection of SP and non-SP cells into athymic mice. SP cells were found to comprise 0.9% of the population and demonstrated the ability to self-renew and differentiate into non-SP cells. Only SP-derived cells were found to have significantly formed tumors compared to non-SP and unsorted cells. In addition, all animals injected with SP cells presented with large metastases in the liver and lymph nodes, whereas animals injected with non-SP cells showed only one animal with liver metastases, and two out of ten animals presented with lymph node metastases. When L3.6pl cells are cultured with increasing concentrations of gemcitabine, the proportion of SP cells, ABCG2 transporter, and CD24 cells were significantly enriched. Some overlap was found between SP cells and CD24+ cells, a previously identified CSC marker; however, there was no overlap found with CD133+ cells [6]. Further work to examining overlap of SP cells with other CSC cell surface marker characteristics may help unite some of the distinct putative pancreatic CSCs being investigated.

Cell Signaling Pathways in CSCs

Defined genetic alterations have been identified in pancreatic cancer; the most notable is an activating mutation in K-ras in over 90% of cases [45]. In addition, a significant number of pancreatic tumors have lost tumor suppressor activity in $p16^{INK4A}$, SMAD4, and p53. Determining the involvement of these genes in the temporal development of premalignant and malignant pancreatic lesions is evolving with the use of genetically engineered mouse models of pancreatic cancer and microdissection of human PanIN lesions. At this time there is not a clear link between these genetic mutations and the phenotypic appearance and behavior of pancreatic cancer cells and CSCs, including their cell surface markers. Several studies have noted alterations in developmental cell signaling pathways that are associated with pancreatic cancer development and progression.

Sonic hedgehog (SHH) is a developmental morphogen in humans that has a critical role in embryogenesis, including normal pancreas development. It is well established from in vitro and in vivo evidence that the SHH signaling pathway is aberrantly reactivated in pancreatic cancer [46–51]. There are studies suggesting SHH is also one of the mediators in pancreatic CSCs [47, 51]. SHH signaling is initiated by the binding of its ligand, namely, SHH, Indian hedgehog, or Desert hedgehog to its receptor Patched which then interacts with Smoothened (SMO). This leads to an intracellular cascade that results in activation and nuclear translocation of the Gli family transcription factor Gli1. Gli transcription factors turn on genes in the nucleus that promote cellular proliferation, cellular survival, stemness, and cell fate determination in a variety of organs [48]. Recent evidence indicates that the Gli genes have a critical role in normal pancreas development and that this dysregulated SHH signaling plays some role in pancreatic cancer [49]. Reverse transcriptase polymerase chain reaction has found that SHH is increased 46-fold in CD44+ CD24 + ESA+ pancreatic CSCs compared with normal pancreatic epithelial cells [46]. Singh et al. [47] identified downstream targets of the Gli genes that regulate cellular

proliferation and survival in pancreatic CSCs by using a small molecule inhibitor of SHH signaling, GDC-0449. GDC-0449 induced significant cell death in pancreatic CSCs isolated from three pancreatic cancer cell lines and decreased expression of SHH signaling components Gli1, Gli2, Patched-1, Patched-2, SHH and Smoothened, Gli-DNA binding, and Gli-luciferase reporter activities. GDC-0449-induced changes in gene expression and apoptosis were blocked by Gli1 plus Gli2 shRNA, thus pointing a role of Gli for cellular proliferation and survival in human pancreatic CSCs [47].

Initially, there appeared to be a correlation between in vitro, in vivo, and clinical data in regard to SHH. Overexpression of SHH and its downstream effector, Gli1, is associated with a poor overall survival of pancreatic adenocarcinoma patients [52]. However, a pilot clinical trial with an SHH inhibitor alone or in combination with gemcitabine failed to improve clinical outcomes in pancreatic cancer patients [53]. Similarly, strategies that target signaling pathways overexpressed in more differentiated pancreatic cancer cells alone or in combination with conventional cancer therapeutics have disappointed in clinical trials [54]. It is clear that the SHH pathway plays a role in pancreatic CSC signaling; however, based on the above evidence, this pathway is not sufficient for maintenance of this cell population in isolation, and future therapeutic strategies need to simultaneously target additional regulatory pathways in differentiated cancer cells as well as pancreatic CSCs.

The Notch pathway controls important cellular processes including stemness, differentiation, proliferation, and survival [55]. In addition, Notch pathway activation is described for many human cancer types, including lung, colorectal, breast, and pancreatic cancer [56–58]. In mouse models for pancreatic cancer, the Notch signaling pathway has shown to be important, where inhibition of Notch signaling by a γ-secretase inhibitor (GSI) completely blocked tumor formation [58]. There is also evidence suggesting inappropriate activation of Notch signaling could be an early event leading to accumulation of undifferentiated precursor cells in pancreatic cancers and promotes survival of CSCs [59]. Using primary human pancreatic xenografts, Abel et al. (2014) demonstrated that the CSCs (CD44+ CD24+ ESA+) had upregulation of the Notch pathway components, including Notch 1–3, Hes1, Jag2, and DDL1. Inhibition of the Notch pathways with a gamma-secretase inhibitor R0492907 or Hes1 shRNA reduced the percentage of CSCs and tumorsphere formation. Furthermore, in vivo treatment of orthotopic pancreatic tumors in NOD/SCID mice with the gamma-secretase inhibitor MK-0752 also blocked tumor growth and reduced the CSC population in the tumors [60]. Similarly, Wang et al. found higher Notch1 expression in pancreatic CSC compared with the non-CSC population in L3.6pl cells [61]. Ji and colleagues reported that $CD44^{+}/CD133^{+}$ expressing pancreatic CSCs contain high levels of Notch1 and Notch2 and that restoration of miR-34 downregulates both receptors [62].

DLL4 is an important component of Notch-mediated stem cell self-renewal and vascular development. Yen et al. investigated the contributions of DLL4 in tumor cells and in the host vasculature and stroma in a panel of xenograft models derived

from pancreatic cancer patients by treating the mice with neutralizing antibodies against human and mouse DLL4. Anti-DLL4 was found to reduce CSC cell frequency as a single agent and in combination with gemcitabine. It was found that the effect on CSCs in xenograft experiments was due to targeting DLL4 expressed on human tumor cells and not mediated through inhibition of DLL4 in the host stroma and vasculature [63].

Ponnurangam et al. identified a pharmacological agent, Quinomycin, targeting the Notch signaling pathway in a mouse model of pancreatic cancer [64]. Nude mice carrying tumor xenografts were administered Quinomycin, and it was found that treatment with the compound significantly inhibited tumor xenograft growth, coupled with significant reduction in the expression of CSC markers and Notch signaling proteins. Moreover, Quinomycin affected pancreatosphere formation and decreased the expression of CSC marker proteins DCLK1, CD44, CD24, and EPCAM. Furthermore, levels of Notch 1–4 receptors; their ligands Jagged1, Jagged2, DLL1, DLL3, and DLL4; and the downstream target protein Hes-1 were reduced. Ectopic expression of the Notch intracellular domain (NICD) partially rescued the cells from Quinomycin-mediated growth suppression. Together, these data suggest that the Notch signaling pathway is an integral component of CSC survival in pancreatic cancer [64].

Bmi1 is a member of the Polycomb group family of proteins that has been has found to be important in oncogenesis in multiple solid tumors [65, 66]. It has also been shown to be important for maintenance and self-renewal of normal stem cells. Proctor et al. noted overexpression of Bmi1 in the cancer stem cell compartment in primary human pancreatic cancer xenografts and that tumorspheres demonstrate high levels of Bmi1 compared to bulk tumor cells [67]. Silencing of Bmi1 with shRNA in CSCs derived from primary human pancreatic cancer xenografts resulted in smaller tumor development in NOD/SCID mice and decreased CSCs self-renewal. This study suggests a role for Bmi1 in the regulation of pancreatic CSCs that warrants further investigation.

There is increasing interest in the role of microRNAs (miRs) in CSC biology in multiple tumor types, including pancreatic cancer [62, 68]. miRNAs are small noncoding RNAs involved in the regulation of gene expression at the posttranscriptional level by binding to the 3′-untranslated regions or the open reading frames of target genes, which then leads to either repression or degradation of mRNA [69]. miRs can be classified functionally as oncogenic, if they are upregulated in tumor cells, or tumor suppressor miR if they are downregulated in pancreatic cancer. There is some evidence to support miR contribution to initiation, propagation, and regulation of EMT in CSCs [70]. Clinically, miR-21 expression was shown to correlate with the clinical outcome of pancreatic cancer patients [71]. In addition, overexpression of miR-1246 was shown to be associated with chemoresistance and stemness in pancreatic cancer cells in vitro. In vivo it was found that miR-1246 could increase tumor-initiating potential and induced drug resistance [72]. As the role of miR in stem cell biology continues to evolve, future therapeutic strategies may focus on regulating the miRNA profile in CSCs (Fig. 2).

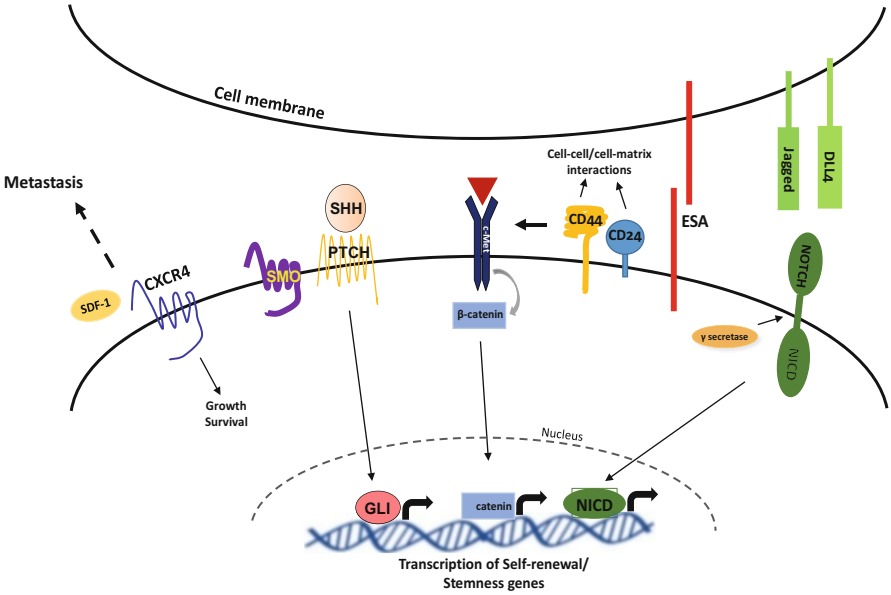

Fig. 2 Signaling pathways in pancreatic cancer stem cells. Cell surface markers ESA, CD24, and CD44 promote cell-cell or cell-matrix interactions, and CXCR4 and c-Met respond to secreted ligands to promote cancer cell migration, invasion, proliferation, and survival. Developmental pathways, such as β-catenin and Notch, are highly active in pancreatic CSCs and could be activated by canonical stimuli or oncogenes, such as c-Met. These pathways stimulate the expression of genes that regulate stem cell properties, such as self-renewal

Altered Metabolism of Pancreatic CSCs

The extensive desmoplasia that accounts for the bulk of the tumor in pancreatic cancer reduces availability of oxygen and nutrients, making pancreatic tumors one of the most hypoxic solid tumors. To maintain proliferation under these growth-restricting conditions, tumor cells undergo marked alterations in cellular metabolism. The oncogenic KRAS mutation is invariably present in most pancreatic tumors and increases glycolysis to generate macromolecules such as amino acids, nucleotides, and fatty acids. Oncogenic KRAS has also rewired glutamine metabolism and increase autophagy and micropinocytosis to provide nutrients for the rapidly proliferating tumor cells [73–77]. Additionally, intratumoral hypoxia also favors the stabilization of hypoxia-inducible factor 1 α (HIF1α) which favors the glycolytic phenotype of pancreatic cancers [78]. However, unlike their rapidly proliferating counterparts, pancreatic CSCs differ in their utilization of glucose and have increased dependence on mitochondrial metabolism and oxidative phosphorylation (OXPHOS). Recent work by Sancho et al. observed that $CD133^{+}$ pancreatic cancer stem cells have increased dependence on OXPHOS and hence have increased sensitivity to the mitochondrial complex I inhibitor, metformin [79]. However,

in vivo treatment of PDX mice with metformin subsequently generated tumors resistant to the drug. The authors further identified that this resistant population ($CD133^+$ $Mito^{low}$) had an intermediary metabolic phenotype with increased glycolysis while maintaining OXPHOS. Notably, these CSCs resembled CSC with MYC activation and PGC1α (peroxisome proliferator-activated receptor-γ coactivator 1) inhibition. The authors concluded that the interplay of MYC and PGC1α levels regulated the metabolic phenotype of CSC and sensitivity to metformin or PGC1α inhibition [79]. Interestingly, a previous study also observed an increase in CSCs ($CD133^+$, $CD44^+$) in pancreatic cancer cells with ablation of oncogenic KRAS signaling [80]. However, in the absence of oncogenic KRAS signaling, these cells lacked the intermediary population as reported by Sancho et al. [79]. Previous studies have elucidated that PGC1α mediates mitochondrial biogenesis, increased invasiveness, and OXPHOS in melanoma and breast cancers [81, 82]. Hence, in pancreatic cancer, increased PGC1α might not only contribute stemness, but it might also contribute to the high metastatic potential of these tumors.

It is important to note that understanding the metabolic phenotype of pancreatic CSCs is contingent upon the type of stem cell markers utilized to evaluate metabolism, the stage of tumor, and the period of study. Examining other established markers and tumor models will help delineate the precise metabolic heterogeneity in pancreatic CSC.

Therapeutic Targeting of Pancreatic CSCs

CSCs appear to explain many aspects of the neoplastic evolution of tumors, and there is also compelling evidence that they may account for therapeutic resistance. Knowledge of the central role that multidrug-resistant (MDR) transporters play in protecting normal stem cells has added insights that may partially explain treatment failure in cancer stem cells. It is known that MDR transporters protect normal and neoplastic cells, and, as such, it is thought that resting cancer stem cells, which are both the cancer-initiating cell and its source of replenishment under selective pressure, have innate drug resistance by virtue of their stem cell phenotype. Acquired drug resistance in more differentiated cancer cells may contribute to an aggressive phenotype, but it may not be the primary reason for cancer recurrence or spread after therapy [83]. In addition, cancer stem cell resistance to radiation therapy is thought to lie in the enhanced capacity of their DNA repair mechanisms. Bao et al. demonstrated that cancer stem cells contribute to radioresistance in gliomas through preferential activation of the DNA damage checkpoint response and an increase in DNA repair capacity [84]. It was shown that CD133+ cells were enriched after radiation in gliomas in both cell culture and immunocompromised mice. Interestingly, the radioresistance of CD133-positive glioma stem cells can be reversed with a specific inhibitor of the Chk1 and Chk2 checkpoint kinases. Further investigation in pancreatic cancer models will be interesting to determine whether a radioresistant population of pancreatic CSCs exists [84]. Current available therapies for patients work by eliminating bulk tumor cells, as targeting pancreatic CSC remains

investigational at this time. Eradication of CSCs is critical for long-term treatment success, as this subpopulation of cells is capable of reestablishing the tumor after the majority of the bulk tumor cells have been eliminated (Fig. 1). New approaches aimed at debulking existing tumors and eliminating CSCs will likely prevent relapse. A number of agents targeting the PCSC pathway have been shown to be effective against pancreatic CSCs in preclinical studies, and clinical trials utilizing some of these targeting approaches are currently underway.

Inhibition of c-Met with XL184 [31] or Alk-4 and Alk-7 with SB431542 [85] reduces the number of CSCs in tumors and had synergistic effects with gemcitabine to reduce the tumor burden in the mice. Maximum benefit was seen when gemcitabine and SB432542 were combined with the Smoothened inhibitor CUR199691 which works by disrupting the pancreatic CSC-stimulated stoma to increase drug delivery. Furthermore, CUR199691 synergized with gemcitabine and rapamycin to inhibit spheroid formation in vitro and tumor burden in mice [86]. The integral contribution of the Notch pathway to pancreatic CSCs was previously discussed, as it has multiple points to target for therapeutic intervention. Inhibition of Notch signaling in tumor xenografts with antibodies against the delta-like ligand4 (DLL4) reduced tumor regrowth in mice treated with gemcitabine, and it was also shown to decrease the proportion of CSCs in tumors. DDL4 inhibition also decreased tumorsphere formation [63]. Similarly, inhibition of the Notch signaling pathway with the gamma-secretase inhibitor MRK-003 in combination with gemcitabine in mice decreased tumor growth and CSC proportions [87].

Recently, it has been proposed that the c-Jun NH_2-terminal kinase (JNK) pathway is important for the self-renewal capacity of PCSCs. AS602801 is an orally administered inhibitor of the JNK pathway that is being tested for its immunomodulatory activity in phase II clinical trials examining endometriosis. Okada et al. examined the effects of AS602801 on bulk pancreatic tumor cells and subpopulations of CD133+ cells, as previous reports have implicated JNK in tumorigenesis [88]. In vitro, AS602801 exhibited cytotoxicity against both bulk tumor cells and CSCs derived from human pancreatic cancer, in addition to non-small cell lung cancer, ovarian cancer, and glioblastoma at concentrations that did not decrease the viability of normal human fibroblasts. AS602801 also inhibited the self-renewal and tumor-initiating capacity of cancer stem cells surviving the initial round of AS602801 treatment. Importantly, CSCs in established xenograft tumors were reduced by systemic administration of AS602801 at a dose that was not toxic to mice. These findings suggest AS602801 may be an anti-CSC agent and further investigation of the utility of AS602801 in the treatment of cancers [89] (Table 2).

Conclusion

The discovery of CSCs has enriched the field of cancer biology by introducing new and important concepts. It is clear that CSCs are a distinct subpopulation within bulk tumor cells that are capable of self-renewal and producing differentiated progeny. Only a select subset of cancer cells are tumorigenic, the CSCs, and these cells appear

Table 2 Agents that target pancreatic cancer stem cells

Agent	Mechanism	Tumor model	
XL184	c-Met inhibitor	O/SC/IC	Li, 2011 [31]
SB431542	Alk-4/Alk-7 inhibitor	O	Lonardo, 2011 [90]
Anti-DLL4	DLL4 blocking antibody	O/SC	Yen, 2012 [63]
MRK-003	γ-Secretase inhibitor	SC	Mizuma, 2012 [87]
CUR199691	Smoothened inhibitor	O/SC	Mueller, 2009 [86]
AS602801	JNK inhibitor	O	Okada, 2016 [89]

IC intracardiac metastasis assay, *O* orthotopic xenograft, *SC* subcutaneous xenograft

to be more resilient to standard anticancer treatment than bulk tumor cells. Pancreatic CSCs possess distinct markers, intracellular signaling, and metabolic features that distinguish them from the non-CSC tumor cells. However, more work needs to be done to more fully understand the molecular machinery that regulates self-renewal and therapeutic resistance. There are many promising new targets on the horizon to strategize how to inhibit the growth of pancreatic CSCs. However, questions remain to be answered in order to translate this knowledge into "actionable" treatments for patients. Targeted therapy against CSCs in conjunction with conventional tumor debulking chemotherapeutic agents is likely required for optimal outcomes in pancreatic cancer patients.

Cross-References

- ▶ Approaching Pancreatic Cancer Phenotypes via Metabolomics
- ▶ Circulating Tumor Cells
- ▶ Emerging Therapeutic Targets in Pancreatic Adenocarcinoma
- ▶ Hedgehog Signaling Plays a Dual Role in Pancreatic Carcinogenesis
- ▶ Notch Signaling in Pancreatic Morphogenesis and Pancreatic Cancer Pathogenesis
- ▶ The Molecular Pathology of Precursor Lesions of Pancreatic Cancer

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Apoptosis: Signaling Pathways in Pancreatic Cancer Pathogenesis

David J. McConkey and Woonyoung Choi

Contents

Abstract

Conventional and investigational cancer therapies have had little to no effect on the course of pancreatic cancer disease progression. Because apoptosis plays a major role in the effects of conventional chemo- and radiotherapy, it has been widely assumed that apoptotic pathways must be disrupted more frequently in pancreatic cancer than they are in other solid malignancies. However, comprehensive genomic characterizations of primary pancreatic cancers do not support this conclusion. Rather, it appears that one of the recently identified molecular subtypes of pancreatic cancer (quasimesenchymal/basal-like/squamous) that shares similarities with basal-like breast and bladder cancers contains tumors

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that are most likely to be apoptosis sensitive and responsive to conventional chemotherapy. Otherwise it is not immediately obvious how the molecular and genomic properties of pancreatic cancers would be expected to impart apoptosis resistance, providing indirect but strong support for the conclusions that late diagnosis and the extent to which tumor-stromal interactions reinforce apoptosis resistance represent the truly unique challenges to effective clinical control of the disease. This book chapter will provide an update of what has been learned recently about the molecular control of apoptosis in pancreatic cancer and how the information might be exploited in the design of more effective therapeutic regimens.

Keywords
BCL-2 family · IAPs · NFκB · EMT · Stellate cells · Cancer-associated fibroblasts · Subtypes

Introduction

"Apoptosis" is a term that was coined by Andrew Wyllie, John Kerr, and Alistair Currie in 1972 to describe a series of stereotyped morphological alterations are associated with most physiological cell deaths, including programmed cell death during development [1]. These changes include chromatin condensation, nuclear and plasma membrane blebbing, cell shrinkage and detachment from neighboring cells, and specific recognition and engulfment by tissue macrophages. Subsequent biochemical studies demonstrated that apoptosis is usually associated with endogenous endonuclease activation, resulting in the formation of oligonucleosome-length DNA fragments ("DNA ladders") [2]. Parallel chemical mutagenesis experiments in *Caenorhabditis elegans* embryos revealed that developmentally programmed apoptosis in the organism requires two genes, termed *ced-3* and *ced-4* [3], and subsequent work revealed that *ced-3* encodes an aspartate-specific cysteine protease (the first "caspase") [4]. Caspases are also required for apoptosis in mammalian cells [5], and caspases are required for the DNA fragmentation associated with the response [6].

Major insights into the biochemical mechanisms involved in caspase activation came from studies with large volumes of HeLa cell extracts, where investigators isolated three proteins ("apoptosis protease activating factors" or Apafs) that could promote activation of recombinant procaspase-3 when the extracts were supplemented with ATP [7]. Microsequencing revealed that one of the proteins was another caspase (procaspase-9) and a second was the mitochondrial electron transport chain intermediate, cytochrome c. The third (termed Apaf-1) is the mammalian homolog of ced-4. Functional studies revealed that Apaf-1 functions as an adaptor protein, promoting the cytochrome c- and ATP-dependent oligomerization and activation of procaspase-9 [8]. Active caspase-9 then cleaves and activates caspases 3 and 7, the two major mammalian "effector" caspases that initiate cell death in mammalian cells.

These observations catalyzed an aggressive investigation of the biochemical mechanisms that control cytochrome c release from mitochondria during apoptosis. Work from several laboratories demonstrated that pro- and anti-apoptotic members of the BCL-2 family are centrally involved. BCL-2 was originally cloned as an oncogene that is located at the t(14;18) translocation that serves as the hallmark feature of follicular non-Hodgkin's B cell lymphomas [9, 10]. Subsequent work revealed that BCL-2 acts to suppress apoptosis [11] and that it is structurally and functionally related to another molecule (BCL-X_L) that also inhibits apoptosis [12]. Investigators showed that the protein localizes to mitochondria [13] and that it binds to structurally related polypeptides that promote cell death [14]. The BCL-2 family is now known to consist of multiple death inhibitors (i.e., BCL-2, BCL-X_L, MCL-1) and death promoters (Bax, Bak, Bad, Bid, Bim, etc.), and that the death promoting members of the BCL-2 family can be further divided into two subfamilies ("multidomain" and "BH3-only") based on the number of domains they share with BCL-2 and the other death inhibitors [15]. Cytochrome c release occurs when activation of a member of the BH3-only subfamily induces Bax and/or Bak to form pores in the outer mitochondrial membrane [16], and the anti-apoptotic members of the family inhibit pore formation by binding to and neutralizing pro-apoptotic members of the family [15]. Interestingly, BH3-only proteins are activated by different environmental cues, and specific BH3-only proteins bind preferentially to specific anti-apoptotic members of the BCL-2 family [17]. These properties of the BH3-only proteins allow for different cell types to display different sensitivities to upstream apoptotic regulators.

The inhibitor of apoptosis proteins (IAPs) make up a second family of polypeptides that play central roles in regulating caspase activation [18]. Originally identified in baculoviruses, the IAPs can directly bind to and inhibit certain caspases, thereby preventing cell death [18]. There is good consensus that X-linked inhibitor of apoptosis protein (XIAP) is the most potent direct inhibitor of caspases, although others (including survivin and the cIAPs) can also block cell death [18]. Parallel studies in *Drosophila* and mammalian cells showed that the death inhibitory activities of the IAPs are under the control of another family of proteins. These polypeptides, including Reaper, Hid, and Grim in *Drosophila* and Second Mitochondrial Activator of Caspases (SMAC) in mammals, directly bind to and neutralize the IAPs, releasing bound caspases to allow them to participate in induction of apoptosis [18]. The sequestration of SMAC within mitochondria places it under some of the same BCL-2 family-dependent mechanisms that control cytochrome c release.

Apoptosis is initiated by a variety of different kinds of intracellular stress and/or by aggregation of surface receptors known as "death receptors" – extrinsic pathway. The most familiar death receptors are the type 1 receptor for tumor necrosis factor, Fas, and the receptors for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which are known as death receptors 4 and 5 (DR4 and DR5). Following ligand-induced trimerization, death receptors recruit an adaptor protein known as Fas-associated death domain (FADD), which binds to and activates caspases 8 and 10 via oligomerization [19]. In lymphocytes caspase-8 activation is sufficient to cause downstream proteolytic processing and activation of effector caspases to cause

cell death. However, in most other cells (termed "type II" cells), the signal must be amplified via the mitochondria for cell death to proceed efficiently [20]. This amplification is also mediated by active caspase-8, which cleaves the BH3-only protein Bid, producing a functionally active form of the protein (tBid) that translocates to the mitochondria and promotes Bax/Bak activation and cytochrome c release [21]. It has been determined that XIAP dictates whether or not a given cell belong to the "type II" category [20]. Although its name implies otherwise, few cancer cells are actually sensitive to TNF-induced apoptosis because TNF usually activates pro- and anti-apoptotic signals simultaneously. The anti-apoptotic signal is dependent on the transcription factor nuclear factor kappa B (NFκB), and inhibitors of NFκB are powerful sensitizers to TNF-induced apoptosis [22].

Apoptosis is tightly linked to proliferation and the molecular mechanisms that control cell cycle progression. Studies focused on the Myc oncogene demonstrated that it promotes both cell cycle progression and apoptosis and that the outcome is determined in part by the presence or absence of exogenous tissue-specific growth factors that are required for maintenance of survival [23]. In normal cells, oncogene-associated apoptosis sensitization involves the tumor suppressor $p19^{ARF}$, which is upregulated by oncogenes and functions to promote accumulation of p53 by shuttling its physiological inhibitor (mdm2) to the nucleolus. TP53 is also required for DNA damage-induced apoptosis in immature thymocytes [24, 25] and oncogene-sensitized normal fibroblasts or epithelial cells [26], observations that served as the basis for the hypothesis that tumor-associated inactivating *TP53* mutations and deletions impart resistance to conventional chemo- and radiation therapy in cancer patients. However, subsequent work in patients with solid tumors have largely overturned this hypothesis. Rather, it appears that p53's more potent effects as an inhibitor of cell cycle progression override its ability to promote DNA damage-induced apoptosis in epithelial cancers. The strongest support for this idea has come from experiments in preclinical mouse models of breast cancer, where stable chemotherapy-induced tumor regressions were only observed in tumors that lacked wild-type p53 [27]. TP53 inactivation was also associated with more clinical benefit in breast cancer patients treated with neoadjuvant chemotherapy in the I-SPY clinical trial [28].

Cell-Autonomous Mechanisms of Apoptosis Resistance in Pancreatic Cancer

There is the sense that some human cancer cells are intrinsically more apoptosis sensitive than others, and if they could be identified prospectively, patients with apoptosis-sensitive tumors could be more aggressively managed with conventional therapies. Recent work has defined the mechanistic basis for intrinsic apoptosis sensitivity [29]. Cancer cells can be "primed for death" because one or more of the anti-apoptotic members of the BCL-2 family are bound constitutively to pro-apoptotic BH3 proteins [29]. This creates vulnerability to any further increase in BH3 protein availability, and this "primed" state has been linked to clinical

response to specific BCL-2 family small molecule inhibitors and conventional chemotherapy [30]. Developing methods to identify these vulnerabilities in pancreatic cancer could be used to identify the patients who will benefit the most from cytotoxic therapies. In addition, these approaches might also allow for the identification of the mechanisms that cause intrinsic resistance and strategies to overcome them. Active nuclear factor kappa B (NFκB) and epithelial-to-mesenchymal transition (EMT) are two such mechanisms that appear to play particularly important roles.

Role of NFκB

Although pathway analyses have implicated "apoptosis" as one of the pathways that is commonly disrupted in pancreatic cancers [31], recently completed comprehensive genomic characterizations of primary human pancreatic cancers have failed to identify high-frequency mutations or copy number alterations that would be expected to directly impart apoptosis resistance (like the t(14;18) chromosomal translocation does in non-Hodgkin's lymphomas) [32]. However, there are indirect apoptosis resistance mechanisms that appear to be particularly relevant to the disease. The one that has been studied the longest involves the inflammation-associated transcription factor, NFκB, which has been widely implicated in the maintenance of cell survival [33]. NFκB is constitutively active in a majority of human pancreatic cancer cell lines and primary tumors [34], and NFκB inhibitors sensitize pancreatic cancer cells to TRAIL- and chemotherapy-induced apoptosis [35, 36]. Several of the central regulators of apoptosis are direct transcriptional targets of NFκB, including BCL-X_L and XIAP [36]. *KRAS* mutations play major roles in driving NFκB activation [37] through direct effects and indirect effects on autocrine and paracrine cytokine production [38]. Constitutive PI3 kinase/AKT pathway activation is also involved [39].

The role of NFκB in maintaining chemoresistance has been examined using RNAi to knock down expression of NFκB's p65 subunit in human pancreatic cancer cell lines and xenografts [40]. This panel included cells that were sensitive (BxPC-3, L3.6pl, CFPAC-1) or resistant (mPANC96, Panc-1, MiaPaCa2) to gemcitabine-induced apoptosis at baseline [40]. Strikingly, p65 knockdown induced apoptosis and increased gemcitabine-induced cell death only in the cells that were sensitive to gemcitabine at baseline. In contrast, p65 knockdown had no effect on basal or gemcitabine-induced apoptosis in the gemcitabine-resistant cells in vitro or in vivo [40]. Therefore, while it is clear that NFκB controls the expression of important anti-apoptotic genes in pancreatic cancer cells, it is not clear that NFκB inhibition will be sufficient to overcome intrinsic apoptosis resistance.

Nevertheless, the preclinical observations implicating constitutive NFκB activation in pancreatic cancer have generated enthusiasm for evaluating NFκB inhibitors in pancreatic cancer therapy. Unfortunately, no potent and specific inhibitors are clinically available, but two "dirty" NFκB inhibitors have been evaluated. The first was the proteasome inhibitor bortezomib (Velcade, formerly known as PS-341),

which in preclinical studies PS-341 inhibited the growth of some [41] (but not all) [42] pancreatic cancer xenografts, effects that were associated with induction of apoptosis and inhibition of angiogenesis. However, combination therapy with bortezomib plus gemcitabine [43] or carboplatin (G. Varadhachary, personal communication) failed to produce any clinical benefit in the second line in patients. Aside from their effects on NFκB, proteasome inhibitors have strong cytostatic effects, and these effects may even interfere with apoptosis induced by conventional therapies.

Curcumin was the second inhibitor to enter clinical trials. It is a natural product NFκB inhibitor that also displayed promising activity in preclinical models of human pancreatic cancer [34]. Unfortunately, it appears that curcumin's low water solubility could be a barrier to clinical development. Even though the dose of oral curcumin selected for Phase II studies was high (8 g/day) [44–46], it displayed poor bioavailability [44, 45, 47]. These observations prompted the development of liposome-encapsulated [48] and lipid-mixed formulations [47], which are still undergoing clinical evaluation.

Role of EMT

Epithelial-to-mesenchymal transition (EMT) is an important developmental program that is often reactivated in epithelial tumors as they progress to become metastatic. The hallmark feature of EMT is loss of the homotypic adhesion due to down-regulation of E-cadherin, accompanied by other changes such as loss of cell polarity genes and increased motility and invasion [49]. These changes in global gene expression are mediated by two transcription factors (Zeb-1, Zeb-2) that recruit histone deacetylases to E-box elements within the E-cadherin promoter [49]. Members of the microRNA (miR) 200 family also play important roles in maintaining the "epithelial" phenotype by repressing Zeb-1 and Zeb-2. Recent work has also demonstrated that these transcription factors can drive some of the canonical epigenetic changes (DNA methylation) that are observed during the progression of solid tumors and cells that have undergone EMT share important properties with cancer stem cells [50].

To identify molecular mechanisms involved in gemcitabine sensitivity or resistance, baseline gene expression profiles were obtained with a panel of human pancreatic cancer cell lines selected on the basis of sensitivity or resistance to gemcitabine-induced apoptosis [51]. The results demonstrated that markers of EMT, and in particular expression of Zeb-1, closely correlated with gemcitabine resistance and cross-resistance to cisplatin and 5-fluorouracil. Knockdown of Zeb-1 not only restored E-cadherin expression but also sensitivity to all three drugs. EMT also generates resistance to EGFR inhibitors, as several studies showed that loss of E-cadherin was associated with resistance to the clinical EGFR inhibitors gefitinib and erlotinib in NSCLC, colon cancer, pancreatic cancer, and head and neck squamous cell carcinoma lines [52–56]. Subsequent work demonstrated that Zeb-1 expression was associated with intrinsic resistance to apoptosis [30], and even more

recent work demonstrated that EMT was not as important for pancreatic cancer metastasis as it was for drug resistance [57]. Other studies indicate that EMT can overcome Kras dependency [58, 59], which could have important implications for the development of Kras inhibitors for pancreatic cancer therapy.

Several clinically available inhibitors of chromatin-modifying enzymes reverse EMT in human cancer cells in vitro. Histone deacetylase (HDAC) inhibitors, including vorinostat, restore E-cadherin expression and enhance EGFR inhibitor sensitivity in "mesenchymal" tumor cells [60]. However, HDAC inhibitors also promote p21-associated cell cycle arrest, and this may not be desirable when these agents are combined with conventional chemotherapeutic agents that are more active in cycling cells. It may also be difficult to achieve biologically active concentrations of HDAC inhibitors without producing toxicity when they are combined with gemcitabine and other conventional chemotherapeutics in patients [61]. Inhibitors of the H3K27 histone methyltransferase EZH2 have also been reported to increase E-cadherin expression and gemcitabine sensitivity in vitro [62], and several of them are now being evaluated in clinical trials. It remains to be seen whether they can reverse EMT and promote sensitivity to chemotherapy at clinically achievable concentrations in patients.

The recent identification of molecular subtypes in primary human pancreatic cancers [32, 56, 63] has important implications for interpreting the impact of EMT on drug resistance in patients. One of the subtypes, termed "quasimesenchymal" [56], "basal-like" [63], or "squamous" [32], is similar to the basal subtypes found in breast and bladder cancers [63] and is enriched with EMT biomarkers [56] and gene expression signatures associated with Myc pathway activation [32]. Therefore, they exhibit biological properties associated with both apoptosis resistance (EMT) and sensitivity (Myc). Preliminary analyses of their clinical properties suggest that like their breast and bladder cancer counterparts, they are associated with shorter disease-specific survival in the absence of neoadjuvant chemotherapy [32], but they may also be more chemosensitive than the tumors that belong to the other subtypes [56, 63]. Prospective clinical studies should be designed to examine whether basal subtype membership is really linked to clinical benefit from neoadjuvant chemotherapy.

Tumor-Stromal Interactions and Drug Resistance

It appears that cell-extrinsic rather than cell-intrinsic mechanisms are largely responsible for the therapeutic resistance that is observed in preclinical models and pancreatic cancer patients. Pancreatic cancers are characterized by an extensive fibrotic stromal compartment that plays important roles in cancer biology. Over the last 15 years, investigators have developed better preclinical models that can be used to define the molecular mechanisms that give rise to the fibrotic stroma and mediate apoptosis resistance in the epithelial compartment of the tumor. In early studies, investigators developed strategies to isolate and culture the cancer-associated fibroblasts (CAFs, also known as "stellate cells") that are a major constituent of the

inflammatory stroma in pancreatic cancer [64]. In vitro co-culture experiments have shown that they promote resistance to gemcitabine and radiation in vitro and they enhance tumorigenicity when they are co-inoculated with human cancer cells in orthotopic xenografts in vivo [65]. Subsequent work in genetically engineered mouse models demonstrated that the fibrotic stroma in pancreatic cancers prevented cancer chemotherapeutic agents and potentially immune cells from even entering the tumors [66]. Many reports had documented that paracrine tumor-stromal interactions involving the sonic hedgehog (Shh) pathway are important in pancreatic cancers, and in the initial preclinical study, a Shh inhibitor promoted sensitivity to gemcitabine by reducing fibrosis and increasing drug delivery [66]. However, in subsequent clinical trials, combinations of gemcitabine plus Shh inhibitors did not produce increased drug accumulation, response rates, or survival in patients [67, 68]. This led to the design of more sophisticated preclinical studies in which stromal fibroblasts were conditionally eliminated either by ablating SHH [69] or by using the fibroblast-specific a-SMA promoter to drive thymidine kinase expression and sensitivity to the cytotoxic drug ganciclovir in CAFs [70]. In both cases, this produced more poorly differentiated and high-grade tumors that were associated with shorter disease-specific survival [69, 70]. In these models, CAF depletion either had no effect on gemcitabine sensitivity [70], or it even inhibited gemcitabine-induced tumor growth control [69], even though CAF ablation was associated with increased apoptosis [70]. These results prompted both groups to conclude that CAFs act to restrict (rather than promote) pancreatic cancer disease progression.

Perhaps not surprisingly, more recent studies demonstrated that even the tumor-associated stroma is heterogeneous in pancreatic cancers. One group used bioinformatics to isolate stroma signatures in public gene expression profiling datasets [63]. They concluded that pancreatic tumors contained either “normal” (stellate cell) or “activated” (CAF) signatures and that the latter were associated with more aggressive disease [63]. The stromal signatures were not associated with tumor molecular subtype (quasimesenchymal/basal vs. classical), but the presence of the “activated” CAF signature was associated with shorter disease-specific survival in both subtypes [63]. The concept of stromal heterogeneity was reinforced by another recent study, which showed that inactivation of the TGFβ pathway in tumor cells was associated with more stromal-epithelial tissue tension, STAT3 activation, and more aggressive disease [71]. Importantly, both studies demonstrated that increased tumor macrophage infiltration was also associated with more aggressive disease biology. Clearly future studies will need to focus on how other aspects of stromal heterogeneity, including differences in cell types and matrix proteins, contribute to the clinical characteristics of these tumors.

Although these observations seem to make the impact of stromal biology on apoptosis even more difficult to understand, some general conclusions and hypotheses can be advanced. As discussed above, apoptosis sensitivity is associated with cell cycle progression, and ablation of stromal fibroblasts generated more undifferentiated, high-grade tumors that proliferated more rapidly [69] and exhibited higher rates of spontaneous apoptosis [70]. Therefore, the results are consistent with the overall idea that the shorter disease-specific survival observed in these models

was not due to apoptosis resistance but rather to more rapid proliferation and progression. It seems likely that more aggressive combination chemotherapy (perhaps including a platinum agent with gemcitabine) would produce more benefit in the ablated tumors versus controls. It would also be interesting to know whether the presence of "activated" fibroblast signatures was associated with more or less benefit in patients with basal tumors (one would expect less). Larger studies are required to address this question.

Conclusions

The completion of large genomic studies and the development of new tools to visualize cancer at the single cell (and cell-free) levels provide an unprecedented opportunity to design new tools for the early detection and treatment of pancreatic cancers. The scientific approach to these problems is also changing, with more emphasis on whole genome as opposed to single gene or pathway analyses and the design of preclinical models (organoids, PDX models, GEMMs) that better capture crucial elements of human disease. It has become clear that apoptosis sensitivity and resistance are not binary states but represent a continuum and predicting therapeutic outcome will require not only knowledge of baseline tumor characteristics but also an understanding of how tumors adapt and evolve in response to cytotoxic stress. Both of the tumor-autonomous apoptosis resistance mechanisms highlighted in this chapter (NFκB activation and EMT) are induced by stress, so future studies must take inducible resistance into account. The tumor-associated stroma also adapts in response to stress, and it will be important to measure the dynamics of these changes in preclinical models and clinical trials in patients.

Progress can also be accelerated by exploiting neoadjuvant clinical trial designs. Past experience in patients treated with neoadjuvant chemotherapy for breast or bladder cancers demonstrated that pathological downstaging predicts for disease-specific survival, which means that candidate regimens can be screened for clinical activity much more rapidly in the neoadjuvant setting. Just as importantly, neoadjuvant studies allow for the collection of matched tumors before and after therapy, which enables the visualization of tumor adaptation. Preclinical models can be powerful tools for mechanistic interrogation but do not rival primary patient tumors as a resource for initial discovery.

Cross-References

- ▶ Chemotherapy for Advanced Pancreatic Cancer
- ▶ EGFR (ErbB) Signaling Pathways in Pancreatic Cancer Pathogenesis
- ▶ Emerging Therapeutic Targets in Pancreatic Adenocarcinoma
- ▶ Development of Novel Therapeutic Response Biomarkers
- ▶ Hedgehog Signaling Plays a Dual Role in Pancreatic Carcinogenesis

- ▶ Neoadjuvant Chemoradiation for Operable Pancreatic Cancer: The Importance of Local Disease Control
- ▶ Neoadjuvant Chemotherapy in Pancreatic Cancer
- ▶ Pancreatic Cancer Stem Cells
- ▶ Precision Medicine Based on Next-Generation Sequencing and Master Controllers
- ▶ Smad4-TGF-β Signaling Pathways in Pancreatic Cancer Pathogenesis

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EGFR (ErbB) Signaling Pathways in Pancreatic Cancer Pathogenesis

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Abstract

The epidermal growth factor receptor (EGFR/ErbB) signaling axis influences the development, maintenance, and disease of tissues throughout the body. Effects have been demonstrated on normal cell proliferation, migration, differentiation, adhesion, and apoptosis in pancreas as well as heart, muscle, nervous system, and a wide variety of organ epithelia. In addition, alterations in the epidermal growth factor (EGF) pathway, including overexpression of the ErbB family of receptor tyrosine kinases, mutations in downstream mediators (e.g., Ras), as well as aberrant signaling, are present in the vast majority of pancreatic and other solid tissue tumors. The importance of the ErbB signaling axis to cancer is illustrated by the number of articles and reviews published on this topic to date (>20,000 and >3000, respectively). In line with the importance of ErbB signaling to cancer, several anticancer therapies have been developed targeting various parts of the ErbB signaling axis and are currently in use, with more undergoing intense development and investigation. Presently, the NIH currently cites an extensive list of clinical studies of ErbB signaling in cancer.

Keywords

ErbB · Epidermal growth factor · EGF · EGFR · Signaling · Pancreatic cancer · Therapy · Molecular imaging

Introduction

Studies of EGF date back to the 1950s when its roles in gastrointestinal ulcers, and subsequently in cancer, were discovered [1, 2]. EGF is now known as the founding member of the EGF family of ligands. EGF ligands signal through the ErbB family of receptors to alter intracellular protein activity, gene transcription, and cell biological status with respect to proliferation, migration, differentiation, and more.

ErbB signaling has roles in numerous diseases, most notably cancer but also psoriasis, Alzheimer's disease, and schizophrenia [3]. ErbB1 is overexpressed in colorectal, gastric, ovarian, renal, prostate, cervical, brain (including glioblastoma multiforme, GBM), non-small cell lung cancer (NSCLC), and squamous cell head and neck cancer. ErbB2 is a potent inducer of neuroblastoma and metastatic mammary tumors in rats and is overexpressed or mutated in many human cancers including breast, brain (including GBM), and NSCLC. In mouse, an increase in ErbB signaling causes cancers of the pancreas, breast, lung, colon, stomach, ovary, brain, prostate, and kidney.

In the pancreas, ErbB signaling affects development and growth of both the endocrine and exocrine pancreas [4, 5], and its receptors influence the development and progression of pancreatic cancer. In fact, ErbB1, also known as EGFR, is overexpressed in 30–90% of pancreatic cancer [6] where neoplastic cells appear to enter the lymph node and establish metastasis to other organs [7]. EGFR has become

a model of translational research that raises the stature of basic science. This is a prime example of how a molecule discovered in the laboratory can transcend the bench and become a therapeutic proof of principle. Moreover, studies on ErbB family members have inspired the birth of other molecular-targeted areas, such as anti-VEGF, anti-TGF-β, anti-c-KIT, and others.

EGF Ligands

EGF ligands have different affinities for the different ErbB receptors [3, 8]. Seven ligands have high affinity for ErbB1 (amphiregulin (AREG), betacellulin (BTC), EGF, epigen, epiregulin (EREG), heparin-binding EGF-like growth factor (HBEGF), and TGF alpha). Of these, BTC, epigen, and EREG also bind ErbB4, as does neuregulin (NRG)-1, NRG-2, NRG-3, NRG-4, and tomoregulin. NRG-1 and NRG-2 also bind ErbB3, as does Neuroglycan C (Fig. 1).

All of the EGF ligands are single-transmembrane glycoproteins with an N-terminal extracellular region and C-terminal cytoplasmic tail [8]. A juxtamembrane extracellular EGF domain is common to all ligands. This EGF domain contains six conserved cysteines, which form three disulfide bonds providing a common secondary structure and allowing interaction with ErbB receptors. EGF is unique in that it has nine repeats of the EGF domain. The presence of AR, BTC, and EPR in syntenic regions of human chromosome 4 and mouse chromosome 5 suggests these ligands

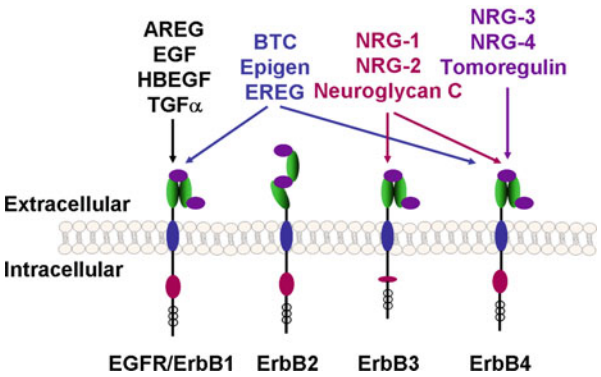

Fig. 1 ErbB receptors and their ligands. The four ErbB receptors with their corresponding high-affinity activating ligands are shown. All four receptors have an extracellular amino terminus with two cysteine-rich (*CR*) domains (*green*) containing the dimerization domain (*DD*) and two leucine-rich ligand-binding domains (*L*) (*purple*). Receptors all have a single-transmembrane domain (*blue*), an intracellular kinase domain (*pink*), and carboxy terminal tail with tyrosine phosphorylation sites (*open circle*). Receptors are shown here as inactivated monomers (*no ligand bound*). In the inactivated state of ErbB1, ErbB3, and ErbB4, the two CR domains are tethered by disulfide bonds (*shaded green*) sequestering the DD so that it is unavailable for dimerization. In contrast, ErbB2 does not bind ligand and has a constitutively exposed DD. ErbB3 is unique in that its kinase domain is inactive. Abbreviations for ligands are defined in the text

arose by a gene duplication event that preceded the divergence of human and mouse [8].

Ligands may signal while membrane bound or as a proteolytically cleaved, soluble, extracellular portion [8]. The cleaved form is generated in a process called ectodomain shedding by the activity of a sheddase (a protease of the matrix metalloprotease (MMP) or a disintegrin and metalloprotease (ADAM) family). The efficiency of cleavage is determined by the sequence at the site of cleavage, the length of the juxtamembrane domain, and availability of particular sheddases, which have preferential activity for specific ligands [9]. Following cleavage, signaling may be autocrine (on the same cell), juxtacrine (on an adjacent cell), paracrine (on a nearby cell), or endocrine (on a distant cell).

EGF Receptors

The receptor family consists of four single-transmembrane glycoproteins, ErbB1 (EGFR, HER1), ErbB2 (HER2, *neu* in rodents), ErbB3 (HER3), and ErbB4 (HER4). The four ErbB receptors share several common functional domains (Fig. 1). The extracellular domain includes the N-terminus, leucine-rich domain (LD) 1, cysteine-rich domain (CD) 1, LD2, and CD2. There is a dimerization domain (DD) in CD1, which is hidden in ErbB1, ErbB3, and ErbB4, but not ErbB2, due to disulfide bonds tethering CD1 and CD2 [3]. These bonds are absent in ErbB2, leaving the DD constitutively available. Intracellular domains include the juxtamembrane domain, tyrosine kinase domain, and C-terminal tail containing tyrosine phosphorylation sites. ErbB3 differs from the other ErbB receptors in that it has an inactive tyrosine kinase domain [3].

Classical activation of ErbB1, ErbB3, and ErbB4 results from binding of a single ligand to a single receptor monomer, inducing a conformational change exposing the dimerization domain within CD1 (Fig. 1) [3]. ErbB2 has no known ligand but does not need one for dimerization since its DD is constitutively exposed. Homo- and heterodimerization follow exposure of the dimerization domain (Fig. 2). Most ErbBs have the highest affinity for ErbB2 as a dimerization partner; however, dimer composition is ultimately a function of both affinity and levels of expression of receptor monomers [3]. Following dimerization, the tyrosine kinase domain of ErbB1, ErbB2, and ErbB4 phosphorylates the C-terminal tail of its dimerization partner [3]. Due to its inactive kinase domain, ErbB3 cannot phosphorylate another ErbB receptor, although another ErbB receptor can phosphorylate ErbB3. Together, these events, initiated by the dimerization of different ErbB family members, can be referred to as the ErbB canonical pathway.

Noncanonical pathways can also activate ErbB receptors. Any activation of ADAM family metalloproteinases, such as activation of G protein-coupled receptors by non-EGF ligands, including endothelin-1, bombesin, thrombin, lysophosphatidic acid, Wnt1, Wnt5, and angiotensin-II, can induce cleavage of EGF ligands and activation of ErbB receptors [3]. Integrins can increase the translation of ErbB2 and ErbB3 and form a complex with ErbB2 and Src resulting in ErbB2 phosphorylation

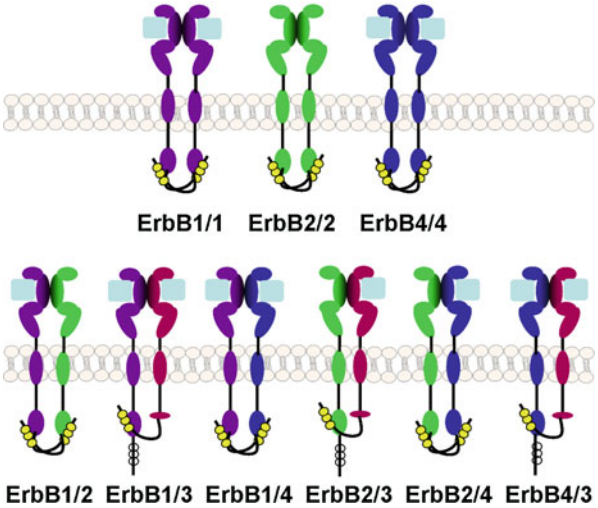

Fig. 2 ErbB Dimers. There are nine possible signaling ErbB dimer combinations. Monomers of ErbB1 (*purple*), ErbB3 (*pink*), and ErbB4 (*blue*) change conformation with ligand binding (*light blue*) such that the DD becomes available and the monomer forms a dimer. Upon dimerization with all ErbB monomers except ErbB3, tyrosines in the C-terminal tail become phosphorylated (*yellow*) by the dimerization partner. ErbB2 (*green*) does not bind ligand and has a constitutively available DD. The kinase domain of ErbB3 is inactive, thus ErbB3 cannot phosphorylate its dimerization partner; however, ErbB3 can be phosphorylated. The phosphorylated tyrosines bind and activate intracellular proteins with SH2 and PTB domains. Shown on top are the possible activated ErbB homodimers (ErbB1, ErbB2, and ErbB4) and on bottom the activated heterodimers

and activation. In addition, ECM proteins [10], cell adhesion proteins, proteins related to the immune response, and several poxviruses [11] utilize the ErbB signaling pathway. Understandably, the identification of noncanonical pathways has elicited significant excitement since several of them help explain processes that were obscure before their discovery.

Post-receptor EGF Signaling

Phosphotyrosines on activated ErbB receptors create binding sites for Grb2 and Src homology 2 (SH2) proteins, activating signaling of the RAS/RAF/MAPK, PLCγ1/PKC, PI3kinase/AKT, and STAT pathways (Fig. 3) [12]. An analysis of the affinity and specificity of ErbB receptors for signaling proteins showed that ErbB1 and ErbB2 are the most promiscuous, with ErbB3 following, and ErbB4 showing the most specificity [13]. Downstream signaling varies depending on the specific ligand bound and the monomer composition of the ErbB dimer [14, 15].

Following signaling, receptors may be dephosphorylated, cleaved, or endocytosed [3]. Dephosphorylation, which results from the activity of phosphatases such as density-enhanced phosphatase-1 (DEP1) and protein tyrosine phosphatase

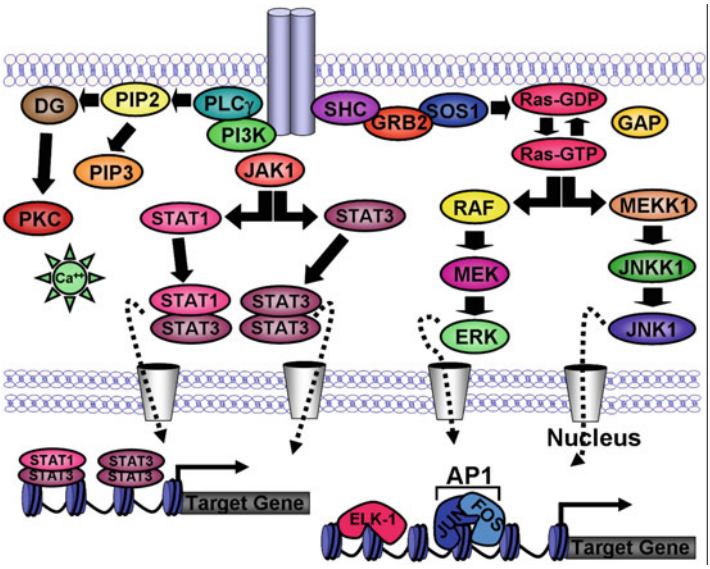

Fig. 3 ErbB signaling pathways. EGF receptor activation initiates a diverse array of cellular pathways via dimerization (represented by the *light blue cylinders* in the cell membrane). Each receptor dimer recruits different Src homology 2 (SH2)-containing effector proteins triggering distinct signaling pathways, culminating in cellular responses such as cell proliferation or apoptosis. The activated receptor complexes with the adaptor protein, GRB2, which is coupled to the guanine nucleotide-releasing factor, SOS1. This GRB2-SOS1 complex can either directly bind to receptor phosphotyrosine sites or indirectly through SHC. As a result of these interactions, SOS is localized in close proximity to RAS, allowing for Ras activation. Subsequently, the ERK and JNK signaling pathways are activated, which ultimately lead to the activation of transcription factors, such as c-fos, AP-1, and Elk-1, that promote gene expression and contribute to cell proliferation. In addition, in response to EGFR activation, JAK kinases activate STAT-1 and STAT-3 transcription factors, contributing to further proliferative signaling. Protein kinase C (PKC) is also activated via phosphatidylinositol signaling (PIP2➔PIP3) and calcium release, which serves as another node of EGF signaling. See text for further details

(PTP) 1B, stops signaling by removing sites for adaptor proteins to bind. Endocytosis may stop or promote signaling by promoting ligand dissociation, lysosomal degradation, and possibly nuclear targeting. Once in lysosomes, receptors remaining bound to ligand are more often degraded, while those dissociated from ligand are more often recycled to the membrane. This may provide a regulatory step to stop signaling preferentially of high-affinity ligand-receptor combinations. One notable exception is ErbB1/ErbB2 dimers, which preferentially escape degradation and are recycled, and thus tend to signal longer. ErbB1 signaling induces several proteins with a negative feedback effect, promoting its own degradation, such as Sprouty-2, LRIG-1, MIG-6/RALT, and suppressor of cytokine signaling-5 (SOC-5).

All ErbB receptors have been found in the nucleus where they may function as transcription factors or cofactors [3]. ErbB receptors contain three clusters of basic amino acids in the juxtamembrane domain with homology to known nuclear

localization sequences. Nuclear localization of ErbB1 causes the upregulation of several cancer-related genes, such as cyclinD1, B-myb, cyclooxygenase-2, and members of the iNOS/NO pathway [16]. ErbB4 undergoes a ligand-dependent proteolytic cleavage of the intracellular domain [9]. However, investigation on the nuclear role of ErbBs is still at its infancy in many organs and certainly underrepresented in the field of pancreatic cancer research. Therefore, this area of research offers a unique opportunity for potential fruitful discoveries that can advance our knowledge on this painful disease.

Signaling via the Canonical EGF-RAS-ERK Pathway

This pathway is of paramount importance for the pathobiology of pancreatic cancer since its alteration, at many levels, associates frequently with this disease [17]. Upon dimerization, the ErbB receptor becomes autophosphorylated at multiple tyrosines within its cytoplasmic domain (reviewed in [12]). The generation of a phosphorylated tyrosine acts as a docking site on this receptor for proteins containing domains similar to a portion of the Src oncogene and thus termed Src homology 2 domains (SH2 domains). The SH2 domain-containing protein, Grb2, binds to the receptor and subsequently recruits the guanine nucleotide exchange factor, SOS (Fig. 3). Another Src homology domain in Grb2, called an SH3 domain, is a proline-binding motif that interacts with many proteins. SOS acts as the guanine nucleotide exchange factor (GEF) for RAS which unloads GDP and binds GTP to become activated (Fig. 4). Inactivation of RAS requires the exchange of GTP for GDP again. This GTP hydrolysis can be accelerated by GTPase-activating proteins (GAPs). Noteworthy, the human genome encodes three *RAS* genes that give rise to four ubiquitously expressed gene products, though only one of them, *K-RAS*, is mutated in more than 90% of pancreatic tumors [17]. This family of proteins is composed of H-RAS, N-RAS, K-RAS A, and K-RAS B (K-RAS A and K-RAS B are splice variants of single gene). The *H-RAS* gene was named such due to its homology to the oncogene of the Harvey murine sarcoma virus, while the *K-RAS* acquired its name from homology to the oncogene of the Kirsten murine sarcoma virus. The *N-RAS* gene does not have a retroviral homolog, but it is identified this way because it was originally isolated from neuroblastoma cells.

The process of Ras activation involves the migration of GTP-bound RAS to the membrane where it recruits RAFs, a group of three serine/threonine kinases (A-RAF, B-RAF, C-RAF) whose regulation is complex and not completely understood. Membrane-localized RAF is activated by multiple phosphorylations and dephosphorylations. Subsequently, RAF phosphorylates two serine residues in the activation loop of MEK1/2 (Fig. 4). MEK is a dual specificity kinase that phosphorylates ERK on both the threonine and tyrosine within a conserved TEY motif in the activation loop. Two isoforms of this enzyme exist, ERK1 and ERK2, which share many functions, though independent knockouts of these proteins in mice suggest that sometimes they may have nonredundant functions in vivo. Activated ERK can phosphorylate more than 100 substrates at various locations in the cell, creating a

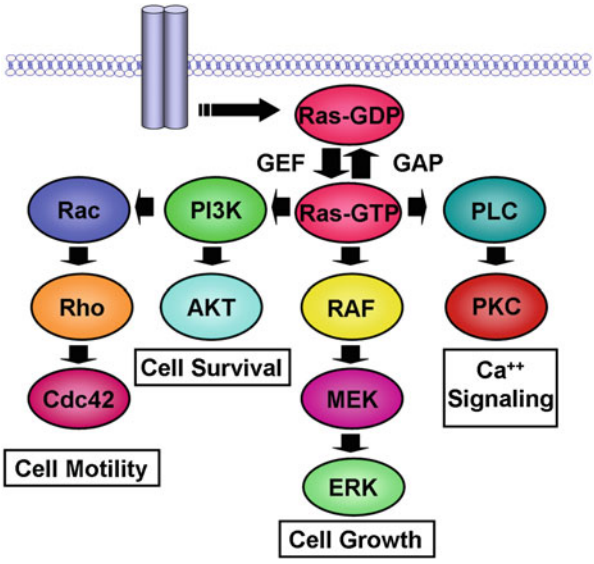

Fig. 4 Downstream RAS signaling. After receptor activation (represented by the *light blue cylinders* in the cell membrane), RAS activation is regulated by the cycle of hydrolysis of bound GTP. The activated receptor signals to a guanine nucleotide exchange factor, such as SOS1 (see Fig. 3), which then ejects GDP from RAS to allow RAS to bind free GTP to become active. Opposing this activation are the GTPase-activating proteins (GAPs), which stimulate the endogenous GTPase of RAS, thereby creating inactive RAS-GDP. Although PI3K can be activated via its recruitment to ErbB receptors, PI3K can also be activated by RAS directly. Activation of PI3K results in not only activation of AKT and its downstream effectors (see Fig. 6) to mediate cell survival but an increase in PtdIns(3,4,5)P3 at the plasma membrane as well. This leads to the activation of the Rho family of small GTPases, Rho, Rac1, and Cdc42 via recruitment of GEFs to the plasma membrane

multidimensional network. Specifically within the nucleus alone, the dimerized form of ERK actively translocates into the nucleus where it phosphorylates many substrates. These substrates include transcription factors that are regulated by MAP kinase phosphorylation, including Elk-1, c-Myc, c-Jun, c-Fos, and C/EBP β, among others. For instance, the phosphorylation of the ETS family of transcription factors, such as ELK-1, which modulate *c-fos* and *c-jun* expression, leads to activation of the AP-1 transcription factor, which is made up of a Fos-Jun heterodimer (Fig. 3). These regulators are able, among others, to regulate the expression of proteins, such as D-type cyclins, which instruct the cell to enter into the G1 phase of the cell cycle. Thus, the MAP kinase pathway represents signals originating from receptors at the cell surface to the nucleus that result in the regulation of gene expression.

Due to the diverse nature of the downstream substrates this pathway acts upon, normally, the process of activation must be tightly regulated. Thus, evidence for negative feedback loops is found at several levels. For example, ERK-mediated phosphorylation of MEK inactivates the pathway. ERK also activates the kinase RSK2, which can inhibit the ERK pathway by phosphorylating SOS. In contrast,

ERK phosphorylation of RAF appears to enhance activation of the ERK pathway. Finally, there are several phosphatases, including the dual specificity phosphatases (DUSPs), which can inactivate ERK either in the cytoplasm or the nucleus. Therefore, due to their incredible regulatory potential, this pathway relies on maintaining tight checks and balances, which, unsurprisingly, when altered, can easily contribute to the development and maintenance of the cancer phenotype.

EGF Signaling via Other Important, Noncanonical Intracellular Pathways

In addition to the MAPK pathway, ErbB can regulate many cancer-associated cell functions by activating other intracellular kinases and their signaling cascades. Although the detailed description of these cascades is beyond the scope of this article, a brief description of pathways will be provided that are among the most important in cancer-associated processes, such as PI3Ks, PDK, AKT, GSK3β, and mTOR. Phosphoinositide 3-kinases (PI3Ks) were originally discovered as enzymatic activities, which transduce signals downstream of several oncoproteins and growth factor receptors, thereby signaling to induce cell proliferation, survival, and migration. These proteins comprise a family of lipid kinases that are classified into three subfamilies according to structure and substrate specificity (reviewed in [18]). The class IA PI3Ks are of the most relevant to this article due to their clear involvement in cancer [18]. This class is divided into two subgroups. PI3K, which acts downstream of ErbB receptors, is composed of both a regulatory (85 kDa) and a catalytic subunit (110 kDa). There are three catalytic isoforms (p110α, p110β, and p110δ) and five regulatory isoforms (p85α, p85β, and p55γ encoded by separate genes and p55α and p50α that are produced via alternate splicing of the p85α gene).

Recruitment and activation of PI3K to Tyr-phosphorylated ErbB receptors occur via an SH2 domain within the regulatory subunit [19] (Fig. 5). Noteworthy, however, PI3K can also be activated by Ras directly (Fig. 4) [20]. ErbB-mediated activation of PI3K within the plasma membrane microenvironment phosphorylates phosphoinositides (PtdIns) at the 3′-OH position of the inositol ring. The most studied product of PI3K activity is PtdIns(3,4,5)P3 from the phosphorylation of PtdIns(4,5)P2. The PtdIns(3,4,5)P3 molecules bind to pleckstrin homology (PH) domains with one of the relevant PH domains in this context which is that of AKT, also known as protein kinase B (PKB). Through this mechanism, AKT localizes to the membrane, where it is activated by phosphorylation on Thr-308 by PDK1 (3-phosphoinositide-dependent protein kinase 1).

Interestingly, PDK1 contains a PH domain with higher affinity for PtdIns(3,4,5)P3 than AKT [21]. This PDK1 PH domain can, in addition, complex to PtdIns(3,4)P2 which is produced by hydrolysis of PtdIns(3,4,5)P3 in the membrane. This mechanism allows the existence of basal levels of activated PDK1; however phosphorylation of AKT at S473 is required for its full activation. This important step is mediated by mTOR (mammalian target of rapamycin) signaling complex 2 or mTORC2. After full activation, AKT phosphorylates several proteins that mediate

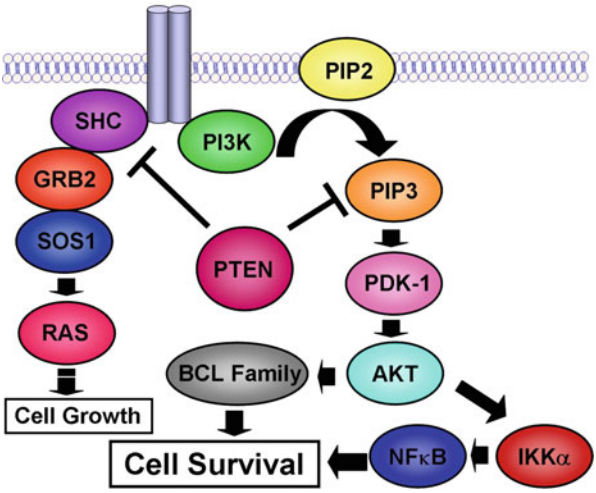

Fig. 5 PTEN regulation of phosphoinositide 3-kinase (PI3K) signaling. Upon activation via receptor signaling (represented by the *light blue cylinders* in the cell membrane), the main substrate of PI3K is phosphoinositide (4,5) bisphosphate (PIP2). Phosphorylation of PIP2 by PI3K generates PtdIns(3,4,5)P3 (PIP3). PIP3 and its 5′-dephosphorylation product, PIP2, are important second messengers that promote cell survival, cell growth, protein synthesis, mitosis, and motility. Cell survival, mitosis, and protein synthesis are all promoted via PI3K-dependent activation of the PDK-1/AKT pathway. Importantly, PTEN is a tumor suppressor gene that is able to dephosphorylate PIP3 in order to regulate this process. Since the activation of AKT is regulated via its phosphorylation by PDK-1, along with integrin-linked kinase (ILK), inactivation of PTEN permits constitutive and unregulated activation of the AKT pathway. In addition to regulating the AKT signaling pathway, PTEN also inhibits EGF-induced SHC phosphorylation to suppress the MAP kinase signaling cascade. Thus, inactivation of PTEN also facilitates the constitutive and unregulated signaling of MAP kinase, lending to an increase in cell growth

the cross talk to other pathways (Fig. 6), such as glycogen synthase kinase 3 (GS3K) and mTOR, regulates the activity of p70 ribosomal S6 kinase-1, and activates eukaryotic translation initiation factor 4E-binding protein-1. These steps are critical to mediate protein synthesis. Thus, together, these cascades of phosphorylations promote growth and survival in many different cell populations. For instance, the activation of PI3K and AKT, as well as the subsequent downstream signaling, promotes survival via a RAS/PIK3/AKT1/IKBKA(IκB kinase-α)/NFKB1 pathway that induces antiapoptotic gene transcription [22].

AKT is negatively regulated by the tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10). PTEN is a lipid phosphatase that catalyzes the reverse reaction of PI3K, by dephosphorylating the D3 position of its lipid products and thereby inhibiting the activation of AKT [22]. Aberrant AKT/PTEN signaling, often found in different human cancers, plays an important role in cancer development, progression, and therapeutic resistance [22].

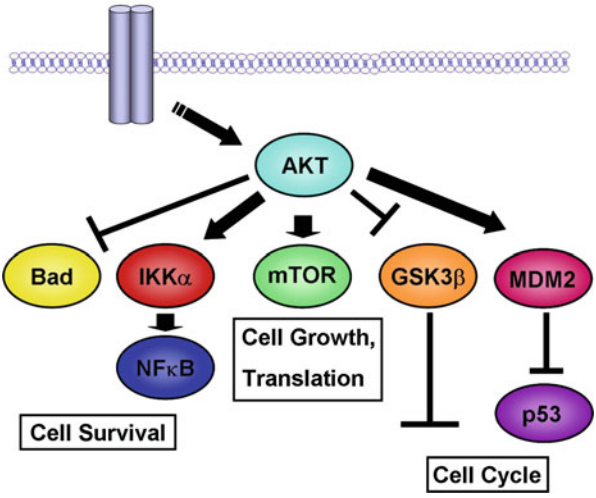

Fig. 6 AKT and its downstream effectors. As shown in Fig. 3, EGFR activation results in direct or indirect activation of PI3K. AKT is located downstream of PI3K and, therefore, functions as a key effector of ErbB signaling. Activated AKT promotes cell survival through inhibition of apoptosis by phosphorylating the Bad component of the Bad/Bcl-XL complex. This phosphorylation causes Bad to dissociate from the Bad/Bcl-XL complex through binding to 14-3-3. In addition, AKT triggers activation of IKK-α that ultimately leads to NFκB activation and cell survival. AKT also regulates cell growth through its effects on the mTOR pathway, as well as cell cycle and cell proliferation through its actions on GSK3β, resulting in inhibition of cyclin D1, and MDM2, thus indirectly inhibiting p53

ErbB can also signal via members of the Rho family of small GTPases, namely, Rho, Rac1, and Cdc42 (Fig. 4). Like Ras, these proteins are activated when bound to GTP and inactive in the GDP-bound state, steps that are mediated by specific GEFs and GAPs, respectively. As mentioned above, receptor activation stimulates PI3K, resulting in an increase in PtdIns(3,4,5)P3 at the plasma membrane. This increased in PtdIns(3,4,5)P3 recruits, via the PH domain members of the Vav family of proteins (Vav1, 2, and 3), which by acting as a GEFs, lead to the activation of Rho, Rac1, and more discriminately, Cdc42 [23]. Vav is not the exclusive GEF for this protein since, for example, several others GEFs, including Sos1, Sos2, and Tiam1, have been shown to transduce the growth signal from the EGF receptor to Rac1 [24]. Small GTPases of the Rho family are involved in a variety of functions in different cells, though they are notorious for their role in cytoskeletal reorganization and cell migration. For instance, Cdc42 controls the assembly of filopodia [25], Rac1 stimulates the formation of lamellipodia and membrane ruffles, and RhoA regulates the assembly of stress fibers [26].

The proteins Diaphanous 1 (Dia1) and ROCK signal the activation of some Rho GTPases to their action on the actin cytoskeleton [27]. Dia1 stimulates actin polymerization and actin bundle formation. ROCK activates myosin to cross-link actin bundles, and, as a result, the formation of actomyosin bridges to induce contractility.

Besides their role in motility, Rho GTPases are also emerging as important regulators of the Wnt-APC-beta-catenin signaling, which is of paramount importance for the regulation of cytoskeletal dynamics, cell adhesion, gene expression, and cell growth (reviewed in [28]). Interestingly, at least some part of this pathway appears to be necessary for pancreatic cancer, since it has been demonstrated that aberrant expression of Vav1 acts as a dominant oncogenic factor in these tumors and its levels correlate with patient survival [29]. Therefore, future studies in this area may uncover additional pathobiological mechanisms as well as therapeutic targets for pancreatic cancer.

Anti-ErbB-Mediated Therapy for Pancreatic Cancer

Therapeutic targeting of pancreatic cancer has proven to be a significant challenge both for researchers and clinicians due to the aggressive biology, resistance mechanisms, and insufficient knowledge in the molecular characterization of the disease. Due to the frequent activation of EGFR signaling in pancreatic cancer, considerable resources have been invested in the development and implementation of new therapies targeting this oncogenic molecule. Currently, the first line of treatment for patients with advanced or otherwise inoperable disease remains gemcitabine alone or in combination with the small molecule tyrosine kinase inhibitor erlotinib, fluoropyrimidine, or an albumin-bound form of paclitaxel, nab-paclitaxel [30, 31]. Two fairly recent phase III trials in highly selective patient populations have shown increased progression-free survival (PFS) and overall survival (OS). The FOLFIRINOX regimen (infusional 5-fluorouracil, folinic acid, irinotecan, and oxaliplatin) and nab-paclitaxel in combination with gemcitabine demonstrated a significant increase of treatment efficacy in comparison with gemcitabine alone [30]. Gemcitabine (difluorodeoxycytidine) is a nucleoside analog that inhibits ribonucleotide reductase, thereby decreasing the deoxynucleotide pool available for DNA synthesis, with resultant strand termination and eventual cellular apoptosis. This drug was explored for clinical advantage over the standard 5-FU because of its apparent antitumor effects. One study showed that gemcitabine was more effective and provided a modest survival advantage over 5-FU [32]. Despite its establishment as the standard for pancreatic cancer treatment for over a decade, the majority of patients still experienced tumor progression within 2.2–3.8 months with a median OS of 5–6.7 months [33]. For this reason, combinatorial treatment strategies are actively being pursued to improve patient PFS and OS. In this regard, since the EGFR pathway works as a well-established oncogenic stimulus for pancreatic cancer, it has rapidly become a good candidate as a target for combinatorial therapies.

There are two categories of ErbB-targeted therapies for cancer, receptor-targeting monoclonal antibodies (mAbs) and small molecule reversible/irreversible tyrosine kinase inhibitors (TKIs) [34]. Monoclonal antibodies bind to the extracellular domain of the receptor, preventing ligand-induced activation. Tyrosine kinase inhibitors bind to the intracellular kinase domain, inhibiting phosphorylation and

subsequent signaling. In general, the monoclonal antibodies developed to date are more specific than the tyrosine kinase inhibitors, yet they also may be immunogenic themselves. Inhibition of EGFR has also been shown to enhance molecular targeting by gemcitabine. In a phase III trial, treatment with gemcitabine plus erlotinib, an EGFR tyrosine kinase inhibitor, compared to gemcitabine alone in 569 randomly assigned patients with unresectable, locally advanced or metastatic pancreatic cancer, exhibited significantly longer OS in patients treated with the combination of gemcitabine and erlotinib [35]. Significant improvement with the combination of gemcitabine and erlotinib was also observed in 1 year survival rates of 23% and PFS with a median survival of 3.75 months in comparison with 17% and 3.55 months, respectively, in the gemcitabine alone group. In fact, the results of these studies prompted the FDA to approve erlotinib for use as a second-line therapy for recurrent, metastatic pancreatic cancer. Subsequently, a phase II study evaluating the combination of gemcitabine with erlotinib established a relationship between the presence of grade 2 or higher skin rash and longer overall survival in patients diagnosed with advanced PDAC [36]. Noteworthy, however, a multicenter phase II trial, known as RACHEL, concluded that dose escalation of erlotinib to the level of skin toxicity did not result in improved survival for patients with metastatic PDAC [37]. One randomized, prospective trial shed some light on gemcitabine alone or gemcitabine plus erlotinib treatment in pancreatic cancer patients with *EGFR* and/or *K-ras* mutations. The results of that study determined that PFS and OS rates were significantly higher in the combination therapy especially in patients with *EGFR* mutations, while *K-ras* mutation status did not play a role in treatment response or survival [38]. Unfortunately, to date, erlotinib has emerged as the only approved targeted therapy in PDAC despite a number of phase II and III trials [39]. Furthermore, most patients have intrinsic resistance to EGFR inhibitors, not responding to this type of therapy, and in those patients who experience tumor response to EGFR inhibitors, the majority will eventually acquire resistance and face disease progression. In fact, tumors have developed resistance mechanisms through the activation of EGFR-independent signaling pathways that are activated downstream of the ErbB family members so as to promote their survival [40]. However, advancing our understanding of specific cellular and molecular mechanisms that promote resistance to these therapies will help to design new strategies to improve this promising type of agents.

Notably, studies in other cancers have paved the way for their testing in PDAC. The majority of the therapeutic agents available for targeting the ErbB family members in PDAC have also demonstrated moderate efficacy in breast and NSCLC, among others [41]. For example, trastuzumab, approved by the FDA for use in breast and gastric cancers, is a humanized IgG_1 that functions by binding to juxtamembrane domain IV of ErbB2/HER2 to inhibit its ectodomain cleavage and ligand-independent dimerization [42]. This mAB against ErbB2 has exhibited antitumor effects in PDAC with high ErbB2 expression in vitro as well as in vivo xenograft models [43]. However, in a multicenter phase II trial using trastuzumab combined with capecitabine, an oral prodrug of 5-FU, PFS and OS did not function favorably compared with standard chemotherapy, even though the therapy was well tolerated [44]. Lapatinib, a small molecule, functions as a reversible,

ATP-competitive tyrosine kinase inhibitor of both EGFR and ErbB2 and received FDA approval in 2006 for advanced or metastatic breast cancer [42]. Interestingly, this TKI in combination with capecitabine has demonstrated the possibility as a tolerable regimen for patients with gemcitabine-refractory PDAC in a recent phase II trial [45]. While the number of enrolled patients was small (n = 17), a subset of these patients displayed clinical benefit from treatment, suggesting that this combination merits further study. Afatinib, another small molecule, irreversibly blocks signaling from EGFR, ErbB2, and ErbB4 by alkylating a single cysteine residue (Cys773 of EGFR, Cys 805 of ErbB2, and Cys 803 of ErbB4) within the ATP-binding pocket and permanently inactivating the kinase [46]. Afatinib received US FDA approval in July 2013 for the treatment of metastatic NSCLC, which has EGFR exon 19 deletions or exon 21 (L858R) substitution mutations [47]. Recently, studies in preclinical models for PDAC suggest that this drug may display activity, alone and in combination with radiotherapy, independent of *K-RAS* status [48]. The combination of mAbs and small molecule kinase inhibitors is expected to produce remarkable antitumor therapeutic efficacy for many cancers, with the optimism that this would include PDAC [49]. For instance, although the mechanism by which the combination of the EGFR-TKI afatinib and anti-EGFR antibody cetuximab confers potent antitumor activity in vivo has not been fully elucidated, the combination appears to have synergistic effects to inhibit phosphorylation of ErbB1/EGFR, ErbB2/HER2, ErbB3, as well as the downstream signaling molecules, Erk and Akt [50]. Furthermore, combinations of two inhibitors from the same category, such as mAbs, are also being explored. As one example, in a preclinical study, the combination of cetuximab and trastuzumab, both mAbs, was found more efficient as first- and second-line treatment than gemcitabine in human PDAC xenografts [51]. This was followed by a phase I/II trial combining cetuximab and trastuzumab in patients with metastatic pancreatic cancer after gemcitabine failure [52]. While 9 out of 39 patients had stabilized disease, the treatment was suspended due to cutaneous toxicities. However, further investigations are warranted to attempt to control some of these side effects. In addition, several of these therapies are also being combined with PI3K/Akt/mTOR inhibitors (e.g., sirolimus), SRC kinase inhibitors (e.g., dasatinib), Ras/Raf/MEK/ERK inhibitors (e.g., selumetinib), JAK/STAT inhibitors (e.g., ruxolitinib), VEGFR inhibitor (e.g., bevacizumab), and IGF-IR inhibitors (e.g., NVP-AEW541) [53], among others. Nevertheless, all of these studies demonstrate that, although not impressive, targeting ErbB family members alone or in combination displays some type of advantage, which, if enhanced through the rational design of future novel combinatorial agents, may be one of the potential therapeutic approaches to fight PDAC. In this regard, since pharmacological manipulation of epigenetic regulators is emerging as a tool that seeks to increase the efficacy of combinations or even antagonize drug resistance, the use of these molecules in combination with ErbB inhibitors is another promising avenue for future exploration.

Overall, more ErbB-targeted therapeutics remain under development and investigation. In fact, this is a difficult, but potentially fruitful area, where hard-core basic science research can generate a conceptual framework for novel drugs via interactions with other academicians in the fields of molecular modeling, crystallography, and synthetic chemistry, as well as pancreatic cancer diagnosis and management

teams. Thus, this pipeline of investigations to take drugs from the bench to the bedside, as demonstrated for EGFR inhibitors, is the paradigm that may be needed in order to defeat this dismal disease.

Several putative predictors of the efficacy of ErbB-targeted therapy have emerged [34]. The presence of skin toxicity (rash) is positively correlated with efficacy and may be indicative of blood or tissue anti-ErbB concentration. Interestingly, immunohistochemical labeling that shows increased ErbB expression is not a good predictor of response, and good responses have been observed in tumors that stain negative for ErbBs [54]. The presence of mutations in ErbBs seems to be a good indicator of efficacy, as well as an increase in *ErbB* copy number. Tumors with an activated K-RAS do not appear to respond well to anti-ErbB therapy [34]. This may explain the modest effects of anti-ErbB therapy in pancreatic cancer, as activated K-RAS in pancreatic cancer is almost universal. However, this type of therapy, for which development and testing remains active, can potentially be further improved by combining the anti-ErbB compounds with inhibitors of other signaling molecules and cascades involved in cross talk with the EGFR pathway, as described below, or additional downstream targets, against which several drugs have been developed, and many are currently in clinical trials.

Cross Talk between EGFR Signaling and Other Major Signaling Pathways and Their Potential Utility for Additional Therapeutic Strategies

The progression and aggressive behavior of many cancers, and in particular pancreatic malignancies, are caused by not only the deregulation of a single signaling pathway but rather the cooperation among several oncogenic and tumor suppressor signaling cascades. In fact, ErbB family members are readily involved in several cross talk events. For instance, in relationship to other tyrosine kinase receptors, EGFR and c-MET converge on the same downstream signaling cascades and, thereby, elicit similar cellular responses [55]. MET has also been shown to interact with EGFR [56], and their cross talk induces proliferation, invasion, and migration [57], all processes that contribute to carcinogenesis. Interestingly, studies in colon cancer demonstrate that activation of MET while treating cells with cetuximab to inhibit ErbB family members results in resistance to the inhibitory effects of this drug [58]. Similarly, activation of ErbB family members confers resistance to MET inhibition in some gastric cancer cells [59], reinforcing the influence of cross talk between these two pathways.

Reciprocal coprecipitation between EGFR and another tyrosine kinase receptor, namely, the IGF1R, has been detected several in cancer cell lines [60]. Congruently, EGFR knockdown decreases the levels of IGF1R via a mechanism that involves increased IGF1R ubiquitylation and degradation [60]. Furthermore, resistance to EGFR inhibitor drugs has been reported to occur, at least in part, through activation of IGF1R signaling [61]. Thus, although not yet shown in pancreatic cancer cells, due to the related GI origins, it would not be surprising that similar phenomena occur

in this malignancy. Therefore, any studies in this regard should take this existing data into consideration for experimental design and interpretation.

ErbB family members can also cross talk with signaling pathways mediated by serine/threonine receptors. Among these molecules, TGF-β receptor family members display both context-dependent tumor suppressive and tumor-promoting activity in pancreatic cancer [62]. Conventionally, signaling by TGF-β cytokines, namely, TGF-β1, TGF-β2, and TGF-β3, is classified by whether they work via Smad-dependent or Smad-independent pathways. A large amount of non-Smad proteins exist, which are known to mediate TGF-β signaling, but are also part of the EGFR signaling cascade, including several MAPK kinases, members of the ras family of proteins, and KLF transcription factors [63, 64]. Notably, it has been shown that TGF-β1 and TGF-β3 can cooperate with ErbB2/HER2 to stimulate cell motility and invasion [65]. Experimentally, for example, metastases are accelerated in a Neu (ErbB2)-induced mammary cancer model with overexpression of active TGF-β1 or the activated form of the type I TGF-β receptor in the mammary glands of bi-transgenic mice [66, 67]. Furthermore, the pro-migratory effect of TGF-β on cells overexpressing ErbB2 is abrogated by inhibition of ErbB2 with trastuzumab, an ErbB2 neutralizing antibody [68]. In regard to pancreatic cancer, experimental work has tested whether concomitant targeting of EGFR and TGF-β signaling pathways could offer a therapeutic advantage to treat this disease [69]. In this study, shRNA-mediated silencing of EGFR in combination with TGF-β sequestration by soluble TGF-β receptor II (TβRII) was utilized to evaluate the effects on colony formation, tumorigenicity in nude mice, and downstream signaling. In addition, any deleterious effects observed by targeting both EGFR and TGF-β in a pancreatic cell line harboring wild-type Smad4 could be counteracted by concomitant targeting of ErbB family members, TβRI activation, and the intracellular src kinase, suggesting a novel therapeutic approach for PDAC [69]. Thus, it is likely that the tumor-promoting activity of TGF-β appears to rely on either the cross talk with ErbB family members or signaling molecules that are shared in common between these two pathways, suggesting that a combinatorial therapy based on inhibiting these interactions may be either additive or synergistic to control tumor progression.

Transactivation of members of the ErbB family can also involve other types of receptors besides tyrosine and serine/threonine kinases, including sonic hedgehog (SHH), Wnt, Notch, and GPCRs. The Hedgehog ligands signal via two multi-transmembrane proteins, named Patched (PTC) and Smoothened (SMO), with PTC serving as the ligand-binding subunit and SMO as the signaling component [70]. The inhibitory effect that PTC has on SMO is released upon binding of the Hedgehog ligand to its receptor PTC, which allows SMO to trigger a signaling cascade that results in activation of GLI transcription factors. The first description of cross talk between EGFR signaling and the SHH pathway was inferred from the finding that these pathways synergize to induce malignant transformation of skin epithelial cells through activation of several members of the MAP kinase pathway [71]. From this original observation, other investigators have been able to demonstrate that this cross talk is also operational in pancreatic cancer cells, among others [72]. Subsequent studies have shown that the cross talk between these pathways can be integrated in the

nucleus, since the SHH-activated transcription factors, such as c-JUN/AP-1, are also co-regulated by EGF [73]. Not surprisingly, SHH-induced cell proliferation involves the cooperation with the EGFR and PKC signaling pathways [74]. Mechanistically, EGF likely influences SHH signaling through ERK-mediated phosphorylation and stabilization of its master transcription factor GLI1 [75], indicating that different bidirectional cross talk between EGFR and SHH signaling pathways may contribute to the malignant transformation of cancer cells. Through the use of mouse models, cooperation between SHH and Ras signaling has been observed during the earliest stages of PDAC formation [76]. Furthermore, inhibition of SHH signaling enhances the antiproliferative effect of the EGFR inhibitor, gefitinib, in pancreatic cancer cells [77]. Thus, the simultaneous targeting of both EGFR and SHH signaling cascades represents a potential treatment strategy for PDAC.

ErbB activation has also been associated in cross talk with Wnt, which binds to frizzled (Fz) receptors and leads to MMP-mediated release of soluble ErbB1 ligands to ultimately transactivate EGFR [78]. EGFR signaling enhances Wnt signaling through direct ERK MAP kinase-mediated phosphorylation of the WNT co-receptor LRP6, which dramatically increases the cellular response to WNT, as well as phosphorylation of β-catenin, which is known to increase cytoplasmic β-catenin concentration via release of β-catenin from membrane-bound complexes [79]. Another less direct yet efficient way to achieve this level of cross talk between both pathways is evidenced by EGFR pathway-mediated downregulation of caveolin-1, which causes a decrease in E-cadherin, transcriptional activation of β-catenin, and enhanced tumor invasiveness [80].

The Notch pathway is recognized to play essential roles during pancreatic development [81]. In the adult organ, Notch signaling is reactivated during pancreatic cancer initiation. However, recent studies have uncovered a role for Notch receptors in the inhibition of PanIN development, a discovery that suggests that in some contexts, these molecules can also work as tumor suppressors. Thus, it is likely that similar to TGF-β, Notch signaling behaves either as an oncogenic or a tumor suppressive stimulus due to the complexity of signaling with multiple receptors, ligands, and downstream mediators. Cross talk between the Notch and EGFR pathways has been observed in several cancer types. While independent inhibition of either EGFR or Notch signaling alone is not sufficient to suppress tumor cell survival and proliferation, simultaneous inhibition of both pathways proves to be an effective combination to eliminate tumor growth, revealing the existence of cross talk between these oncogenic pathways [82]. Notably, a direct relationship has been established between EGF receptor activation and Notch signaling in acinar-to-ductal metaplasia and PanIN formation, as precursor lesions for PDAC [83]. Moreover, Notch signaling synergizes pathways that work downstream of EGFR, such as K-ras, to promote rapid reprogramming of acinar cells to a duct-like phenotype and to induce the initiation of pancreatic carcinogenesis [84]. However, different groups have demonstrated that inhibition of Notch signaling enhances K-ras-mediated PanIN formation [85], again highlighting the context-dependent nature of Notch signaling. In addition, these studies have been done in animal models of pancreatic cancer, which are known to often give rise to contrasting results depending upon their genetic background as well

as the methodologies used to manipulate these pathways [86]. Lastly, while the available models have undoubtedly been technological achievements in the field, their ability to faithfully recapitulate human disease remains limited. Therefore, more work is needed to define whether inhibiting the interaction between the EGFR and Notch pathways would be beneficial or deleterious in humans.

G protein-coupled receptors (GPCRs), similar to ErbB receptors, regulate large signaling networks, which are involved in the development and progression of various cancers [87]. In addition, both of these types of receptors are also being actively studied as preferred pharmacological targets for the treatment of many cancers. Consequently, knowledge of their interaction is of importance, not only for better understanding pathobiological processes but also for learning how to intervene with them for therapeutic purposes. GPCRs are integral membrane proteins with seven transmembrane helices [88]. In humans, there are approximately 800 GPCRs, which are categorized into three main classes (A–C) based on their sequence similarity [88]. In contrast with ErbB receptors, GPCRs lack intrinsic enzymatic activity, but rather couple to heterotrimeric G proteins, which hydrolyze GTP and mediate downstream signaling [87]. In this regard, a sizable amount of publications has documented the interaction of GPCR-mediated signaling cascades and members of the ErbB family of receptors. Indeed, several GPCRs transmit oncogenic signals via the MAPK [89], thereby leading to the regulation of cell growth, cell migration, homing, and metastatic behavior [90]. The first study of this type in the pancreas involved the discovery of cross talk between cholecystokinin (CCK) receptors and EGFR-mediated pathways [91]. Similar to EGFR, CCK receptors are overexpressed in human pancreatic cancer, and their activation by the ligands, gastrin or CCK, stimulates cell proliferation [92]. Interestingly, while PanIN and PDAC development in the K-ras-mediated mouse model is dependent upon the presence and activation of EGFR [93], the pharmacological inhibition of CCK receptors also reduces the number of these lesions, suggesting that these pathways may synergize [94]. In addition to CCK, another neuropeptide, neurotensin, induces rapid and dose-dependent ERK1/2 activation with subsequent stimulation of DNA synthesis in PDAC cells [95]. Beyond these few examples, a plethora of studies have reported that other GPCRs known to cross talk with the EGFR signaling pathway, such as those activated by different GI peptides and chemokines, are expressed in PDAC cells and mediate cell growth and migration [90]. Therefore, potential combinatorial therapies that target GPCRs may enhance the therapeutic index of EGFR-inhibiting drugs and antibodies. Certainly, consideration of cross talk between ErbB family members and other signaling pathways as described will be essential for development of effective therapies.

ErbB-Mediated Molecular Imaging Modalities

ErbB targeting with the goal of generating molecular imaging modalities for tumors is another new area of investigation; however, a detailed description is beyond the scope of this chapter [96]. Briefly, radiopharmaceutical, in particular, labeled humanized

monoclonal antibodies, that specifically target cell surface proteins, including receptors, have been used with the goal of either neoplastic cell ablation (molecular-targeted chemotherapy) or for imaging (molecular imaging) in many tissues. Molecular medicine offers many modalities, including single photon computed tomography (SPCT) or positron emission tomography (PET), which, in combination with MRI and CT, have the potential to give a good definition, anatomical-functional map of a tumor. Several types of antibodies, either whole or as a fragment, are being currently tested against ErbB1 [34]. Some molecules of particular interest due to their potential higher biodistribution and more rapid clearance are the so-called anti-ErbB1 "affibodies." Affibodies are made from three bundle molecules based on 58 amino acids from IgG, and they can bind to their targets at low nanomolar concentration. For example, some of these molecules have been shown to bind to specific tumors in vivo. This is important if the therapy is dependent upon the expression of a distinct cell surface protein. As another approach, near-infrared (NIR) fluorescent labeling is being applied to a mAb against EGFR, cetuximab, as a new tool for fluorescence-guided surgery to visualize tumor margins, as well as metastatic sites in order to achieve precise surgical resection [97]. Molecular imaging techniques to survey other ErbB members, together with EGFR, are also being derived [98]. Furthermore, as these molecular imaging techniques are being actively expanded to other targets [99], this predicts that it could be possible to use a similar approach to cell surface markers that are expressed at the stage of carcinoma in situ (PanIN3) in hopes to push earlier limits of detection. Therefore, molecular imaging offers another wide-open field of study in pancreatic cancer research, and future development in this area has the potential to profoundly impact the diagnosis and therapy of this disease.

It is important to reflect on all of the theoretical frameworks underlying how these signaling cascades work and can be targeted. However, their expected results are "epithelium centric" and do not integrate the potential modulation of ErbB signaling that can occur with other molecules present in the tumor microenvironment. Pancreatic cancer is characterized by a robust desmoplastic reaction, which influences pancreatic cell growth. However, whether other pathways, which are active in the tumor microenvironment, modulate the outcome of ErbB signaling is an area of current investigation. For instance, the existence of cross talk between ErbB1 and integrin signaling has recently been demonstrated to be involved in carcinoma cell invasion and metastasis, which may explain, in part, how inhibitors of EGFR affect malignant disease [100]. However, studies on the role of the desmoplastic reaction in the tumor biology of pancreatic cancer is another rapidly evolving and very promising area of pancreatic cancer research, where most of the discoveries possessing the highest translational potential may occur in the very near future.

Conclusion

Given the central role of ErbB signaling in the regulation of proliferation, migration, and differentiation, it seems likely that therapeutically tapping into this high-level control mechanism can prove useful for pancreatic cancer treatment. It is interesting to

note that ErbB-targeted therapies developed thus far target an ErbB monomer and, as a result all dimers containing that monomer, thus potentially affecting ErbB signaling in complex ways. A more refined strategy with more specific targeting may prove to be significantly more effective. This may be at the level of targeting specific receptor dimers or specific downstream signaling events activated by tumorigenic dimers but not by dimers promoting healthy tissue differentiation. While anti-EGF inhibitors have elicited modest success in the treatment of pancreatic cancer, additional combinations of these agents with small drugs targeting other signaling molecules and cascades involved in cross talk with the EGFR pathway may have synergistic effects and thus offer better therapeutic options. Several important areas of investigation on factors that affect ErbB-mediated signaling, such as noncanonical pathways, the likely modulatory role of factors from the tumor microenvironment, how other cellular pathways are altered under the almost universal presence of oncogenic *K-ras* mutations, and the effects of nuclear ErbBs, remain among the less understood areas of basic research in pancreatic cancer with highly promising translational potential.

Box 1 Key Research Points

- Four ErbB isoforms are differentially expressed in human tissues. They form different types of dimers which then complex with distinct ligands, some found overexpressed in pancreatic cancer (ErbB2-EGF).
- A good understanding of the biochemistry and cell biological processes associated to these receptors is important, not only for the biology but also the pathobiology of pancreatic cancer. Unfortunately, besides ErbB1, little detailed information is available to reconstruct pathways that can be specific to the other isoforms.
- The knowledge of some of these ErbB-mediated pathways such as EGFR is among the best-understood signaling cascades in many organs. The role of ErbB proteins in pancreatic development and cell homeostasis remains an underrepresented area of biomedical research.

Box 2 Future Scientific Directions

- EGFR is the best-studied ErbB receptor isoform, and its contribution to pancreatic cancer, though still far from complete, is better understood. However, pancreatic cancer cells express other combinations of ErbB isoforms, for which the biology and signaling are less known but may be of significant biomedical relevance in this disease.
- More knowledge must be gained on how to modulate the response of anti-EGFR therapy in a manner that can be more beneficial to pancreatic cancer patients. Fortunately, there are numerous signaling nodes that have been identified during the last two decades, which can serve as targets for

(continued)

Box 2 Future Scientific Directions (continued)

combined therapeutic modalities designed to achieve this goal. Many of these targeted therapies have been or continue being developed for clinical use. Combination therapies that target different nodes in this pathway are under active investigation.

- The interaction of pancreatic cancer cells with the tumor microenvironment, which is full of growth factors that bind to ErbB receptors and extracellular matrix proteins that cross talk with these pathways, may provide the basis for future application of this important basic science knowledge to the design of therapy. Therefore, more basic science is needed in this area to inform the development of novel molecularly targeted drugs by focusing on several members of the EGF pathway.

Box 3 Clinical Implications

- Two types of ErbB-targeted therapies exist, which are receptor-targeting monoclonal antibodies (mAbs) and small molecule reversible/irreversible tyrosine kinase inhibitors (TKIs). Currently, erlotinib, an EGFR TKI, remains the only approved molecular-targeted therapy for PDAC.
- The role of EGFR and, in particular, other members of the ERB family of tyrosine kinase receptors, in normal pancreatic molecular cell biology, warrants further investigations. Their translational potential to human disease has not been fully realized. While this has been already an area of fruitful research, due to its translational potential, research on the ErbB pathway and cross talk with other pathways deserve to be further expanded.
- The area of molecular-targeted imaging has extensively benefited on investments on EGFR as a probe. Some of these techniques remain to be refined but represent promising areas of translational research as well as evidence-based medical care.

Cross-References

- ▶ Developmental Molecular Biology of the Pancreas
- ▶ Emerging Therapeutic Targets in Pancreatic Adenocarcinoma
- ▶ Hedgehog Signaling Plays a Dual Role in Pancreatic Carcinogenesis
- ▶ Mouse Models of Pancreatic Exocrine Cancer
- ▶ Notch Signaling in Pancreatic Morphogenesis and Pancreatic Cancer Pathogenesis

► Role of Tumor-Stromal Interactions in Pancreatic Cancer Invasion and Metastases
► Smad4-TGF-β Signaling Pathways in Pancreatic Cancer Pathogenesis

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Hedgehog Signaling Plays a Dual Role in Pancreatic Carcinogenesis

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Contents

Abstract

The hedgehog (Hh) pathway plays an important role in a wide variety of developmental processes including cellular differentiation and tissue patterning. While Hh signaling is a critical component of embryonic development, this pathway is not typically active in most adult tissues. Inappropriate Hh signaling has been associated with several types of malignancies including pancreatic ductal adenocarcinoma (PDAC). In PDAC, the Hh pathway is activated by two distinct mechanisms in the tumor epithelial and stromal compartments. In the stroma, the Hh pathway activity is induced by its ligands in a canonical fashion;

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in tumor epithelial cells its activity is regulated in a ligand-independent manner by known PDAC oncogenic cascades including KRAS, TGFβ, and EGFR signaling. Initial preclinical studies demonstrated that the Hh pathway may be a promising therapeutic target for PDAC. However, Hh inhibition has not been successful in clinical trials of PDAC patients with advanced metastatic disease. Recent reports indicate the Hh pathway may play a dual role in carcinogenesis, acting as an oncogene in early tumorigenesis while switching to a tumor suppressor as the cancer progresses. Current research efforts are aimed at further understanding the role of the Hh pathway in all stages of carcinogenesis and defining the translational value of Hh inhibition in PDAC.

Keywords
Hedgehog · GLI1 · GLI2 · GLI3 · KRAS · TGFβ · EGFR · Tumor microenvironment · Vismodegib

Introduction

The hedgehog (Hh) signaling pathway was first identified in *Drosophila melanogaster* in the late 1970s when Nusslein-Volhard and Wieschaus discovered certain genetic mutations resulted in short larva covered with denticles resembling a hedgehog [1]. An isolated mutation led to identification of the Hh gene coding for the ligand of the pathway. Homologous Hh genes have been identified in many vertebrates including mice and humans. These genes are critical during embryonic development of the neural tube, brain, gut, testis, and limb [2–4]. As a mediator of gastrointestinal development, the Hh pathway is active throughout the epithelial layer of the primitive gut, but it is largely excluded from the region where the pancreas develops [5]. Ectopic Hh expression within the pancreatic domain blocks normal pancreas development and leads to "intestinalization" of the pancreatic epithelium. Aberrant activation of the Hh pathway in the adult pancreas is associated with pancreatic diseases including pancreatitis and pancreatic ductal adenocarcinoma (PDAC) [6–11].

Overview of the Hedgehog Pathway

Hh signaling in mammals has three known ligands, sonic hedgehog (Shh), Indian hedgehog (Ihh), and desert hedgehog (Dhh). All three ligands bind the receptor Patched-1 (Ptch1), a 12-transmembrane receptor. Ptch1 and Smoothened (Smo), a 7-transmembrane (7TM) G-protein-coupled receptor (GPCR), are the core components of the Hh receptor complex. The current paradigm for Hh signaling places the primary cilia as the central cellular location for Hh pathway signal activation in vertebrates (Fig. 1) [12]. In the absence of Hh ligand, Ptch1 binds the shaft of the cilium and inhibits Smo from colocalizing [13, 14]. Localization of Ptch1 at the base of the cilia indicates it may play a role in regulation of proteins that are transported in and out of the primary

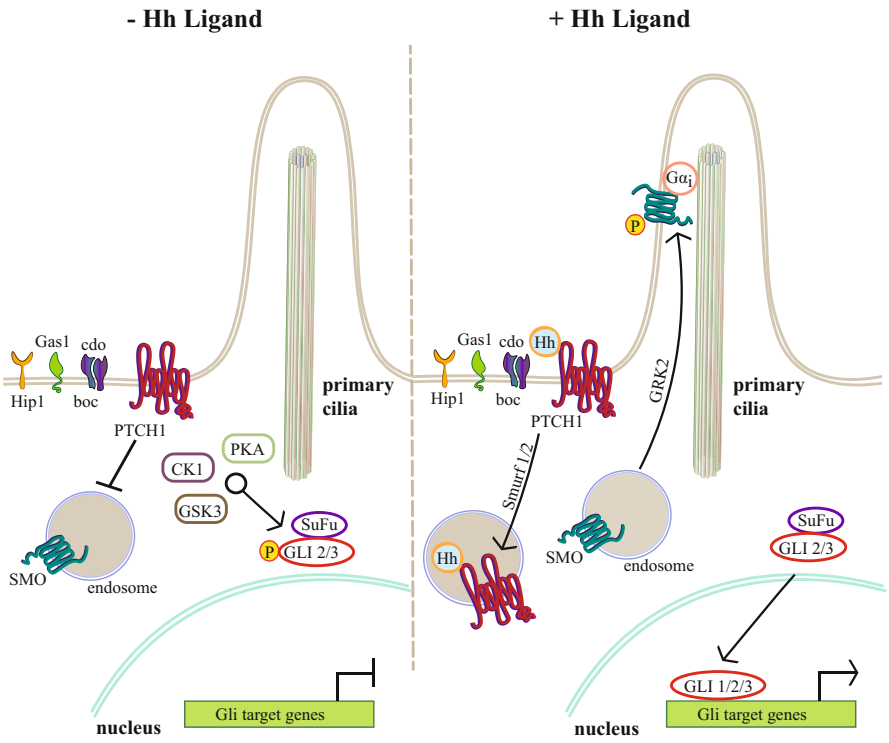

Fig. 1 Hh signaling pathway in the absence and presence of ligand. In the absence of Hh ligand, Ptch1 localizes to the base of primary cilia and inhibits Smo colocalization. Upon Hh ligand binding, Ptch1 is endocytosed through ubiquitination by the E3 ligases, Smurf1 and Smurf2, and subsequently degraded. Coreceptors Gas1, Cdo, and Boc enhance Hh signaling. Following activation, Smo accumulates at the primary cilia and activates downstream signaling of the Hh pathway through the $G\alpha_i$ protein. Smo is phosphorylated by GRK2. Next, β-arrestin 2 binds Smo and promotes its internalization. SuFu binds directly to GLI2/3 in the absence of ligand. PKA, GSK3, and CK1 phosphorylate GLI2/3 to its repressor form. Upon binding of Hh ligand, GLI localizes to the nucleus and binds Hh target genes to activate their transcription. SuFu is ubiquitinated and targeted for degradation

cilia, but the exact mechanism by which Ptch1 inhibits Smo trafficking remains unclear [15]. It was initially hypothesized that there is a direct interaction with Smo, where binding of ligand to Ptch1 induces a conformational change, allowing Smo to dissociate [16]. However, more recent research indicates that Ptch1 does not associate with Smo [17] and may regulate Smo localization through modulating the levels of oxysterol, an oxidized derivative of cholesterol [14], which directly binds Smo and allosterically promotes its localization in the cilium [18]. Upon Hh ligand binding to Ptch1, Ptch1 is endocytosed and ubiquitinated by the E3 ligases, Smurf1 and Smurf2, leading to its degradation [19]. Ptch1 removal from the cilia allows for cell surface accumulation of Smo in the organelle and induction of the Hh intracellular signaling cascade leading to activation of the GLI family of transcription factors, final effectors of the cascade [13].

The GLI family includes three separate zinc finger proteins, GLI1, GLI2, and GLI3. GLI1 and GLI2 are primarily transcriptional activators (although GLI2 has some repressor functions), while GLI3 acts mainly as a repressor of transcription [20]. In addition to its regulation by the Hh ligands, Ptch1 activity is modulated by cell surface proteins, including CAM-related/downregulated by oncogenes (Cdo), brother of Cdo (Boc), growth arrest specific 1 (Gas1), and hedgehog interaction protein 1 (Hip1). Cdo, Boc, and Gas1 enhance Hh signaling; Cdo and Boc bind Hh ligands and facilitate presentation of ligand to the Ptch1 receptor [21], while Gas1 increases the range of the Hh signal during embryogenesis, especially in tissues with low levels of the ligand [22, 23]. Hip1 negatively regulates signaling through sequestration of Hh ligand [24].

It has been demonstrated that G-proteins, β-arrestin, and suppressor of fused (SuFu) play a critical role in mediating Hh intracellular signal following Smo activation. The 7TM structure of Smo utilizes G-proteins for signal transduction, a property shared among most 7TM receptors. However, Smo lacks homology with other GPCRs, and it is missing several GPCR-like features such as the generation of secondary messengers typically associated with G-proteins, like cAMP or calcium [25, 26]. While classification of Smo as a GPCR remains somewhat controversial, Riobo et al. found that Smo activates the $G_{(i)}$ family of G-proteins and this signal is required for GLI activation [27]. However, Smo signaling through G-proteins alone is not sufficient to activate GLI transcription factors. A truncated form of Smo which includes the $G_{(i)}$-activating domain was not capable of activating the GLI reporter, indicating another region of Smo is required for GLI activation. Upon activation, Smo is phosphorylated by the G-protein-coupled receptor kinase 2 (GRK2) [28]. This phosphorylation prevents reassociation of G-proteins with their receptors. β-Arrestins are proteins located in the cytosol that bind phosphorylated 7TM receptors and promote their internalization. Phosphorylation of Smo by GRK2 and interaction with β-arrestin 2 leads to endocytosis of Smo, indicating these proteins play an important role in regulation of Smo signaling [28, 29]. SuFu is a negative regulator of the Hh pathway; its inactivation leads to constitutive Hh signaling [30]. SuFu binds directly to GLI2 and GLI3 proteins, preventing their translocation to the nucleus when no ligand is present [31, 32]. While GLI1 protein functions only as an activator, GLI2 and GLI3 proteins can be transcriptional activators or repressors. In the absence of ligand, protein kinase A (PKA), glycogen synthase kinase 3 (GSK3), and casein kinase 1 (CK1) phosphorylate full-length GLI2 and 3 [33, 34]. This phosphorylation leads to processing of GLI2 and GLI3 to their repressor form and inhibits localization of the GLI-SuFu complex to the nucleus [35]. The phosphoinositide 3-kinase (PI3K)/Akt pathway is an important regulator of PKA. Akt is required for inhibition of PKA-dependent GLI2 inactivation [36].

Upon Hh stimulation, Smo accumulation in the cilia leads to SuFu dissociation from GLI2 and GLI3 in part through the action of the kinesin motor protein, Kif7 [35, 37]. Following dissociation, SuFu is ubiquitinated and targeted for degradation, while GLI2 and GLI3 translocate to the nucleus [35, 38]. One study found that SuFu recruits SAP18, a component of the mSin3-histone deacetylase corepressor complex, to GLI1 promoters, indicating that SuFu may not be degraded and can translocate to the nucleus to act as a co-regulator of GLI [39]. In addition to SuFu

repression of GLI2 and GLI3 activation, the Hh pathway utilizes intraflagellar transport (IFT) to move GLI2 and GLI3 along the cilia toward the nucleus. Disruption of IFT proteins in the cilia prevents active GLI3 formation, indicating the importance of this specialized compartment in Hh signaling [40–42].

GLI proteins activate Hh target genes through binding specific consensus sequences located in the promoter region of these genes, which include molecules responsible for cell fate determination, tissue patterning, proliferation, and transformation as well as components of the pathway [43, 44]. For example, GLI2 transcriptionally activates GLI1, which in turn induces the expression of Ptch1 [45], and other targets of Hh-modulated cellular functions [46]. The kinase DYRK1A phosphorylates GLI1 at a general nuclear localization sequence ("SPS" motif), leading to enhanced nuclear localization [47]. Other protein kinases that have been shown to regulate Hh signaling include protein kinase C-δ (PKCδ) and mitogen-activated protein/extracellular signal-regulated kinase 1 (MEK-1) [48]. In addition, atypical protein kinase C ι/λ (aPKC-ι/λ) plays a role in phosphorylation and activation of GLI1 downstream of Smo. aPKC-ι/λ forms a complex with missing in metastasis (MIM), a centrosome-associated protein that positively regulates Hh signaling and ciliogenesis, and colocalizes to the basal body. Inhibition of aPKC-ι/λ blocks Hh signaling and represses cell growth [49].

Not all Hh pathway signaling is ligand dependent or proceeds through Ptch1/Smo to GLI activation. Subtypes of ligand- and GLI-independent pathways are known as noncanonical Hh signaling. Type 1 noncanonical Hh signaling is Smo and GLI independent. When Shh binds the Ptch1 receptor, Ptch1 may interact directly with cyclin B1 and caspases to inhibit proliferation and induce apoptosis [50]. Ptch1 receptor can also induce apoptosis in the absence of Hh ligand through the adapter protein, DRAL, and caspase-9 activation [51]. Type II noncanonical signaling is Smo dependent and GLI independent through the activation of a Rho signaling [52–54]. In addition, there are several known noncanonical mechanisms for GLI activation independent of Hh ligand in carcinogenesis. Hh ligand expression does not activate the Hh pathway in a large number of tumor epithelial cells, yet GLI1 is still expressed in these cells, indicating noncanonical activation of downstream components of the Hh pathway [55]. In fact, several studies have shown that GLI expression can be activated through Hh-independent mechanisms, particularly in the epithelial compartment through cross talk between GLI and other pathways such as KRAS, TFGβ, and EGFR [10, 43, 56, 57]. For additional details on noncanonical signaling, refer to the Sect. 4.1.

Role of Hedgehog Signaling in the Pathogenesis of Pancreatitis and Tissue Remodeling

In the adult pancreas, the Hh pathway is typically only active in endocrine cells within the islets of Langerhans where Hh signaling regulates insulin production and secretion [58]. However, evidence suggests this pathway is upregulated during exocrine regeneration of acinar tissue following injury in mice, where blockage of the Hh pathway

leads to impaired pancreatic regeneration [59–61]. During normal regeneration, Hh is upregulated and then becomes undetectable once regeneration is complete [59, 62]. Thus, dysregulation of the Hh pathway following repair may play a role in pancreatic diseases associated with tissue injury, such as chronic pancreatitis. As described in other sections of this book, chronic pancreatitis is an inflammatory condition that leads to tissue remodeling and fibrosis of the exocrine tissue. While normal adult exocrine and ductal cells do not express detectable levels of components of the Hh pathway, patients with fibrotic tissue associated with chronic pancreatitis show elevated expression of Shh, Ihh, and Ptch1 [6, 63, 64]. Chronic pancreatitis is a known risk factor for PDAC, indicating that deregulation of the Hh pathway in pancreatitis may play a role in tumorigenesis through reactivation of Hh-GLI target genes. Mathew et al. demonstrated that loss of one *Gli1* allele resulted in impaired tissue repair following pancreatitis leading to an altered stroma [65]. Mice with normal GLI1 expression fully resolved architecture and function in the pancreata after 1 week. Hemizygous loss of *Gli* in fibroblasts was also associated with lowered expression of several immune proteins that regulate immune function during tissue damage and repair, including IL-6, MCP-1, and IL-8, and factors that regulate immune cell migration including M-CSF [65]. In addition, loss of GLI1 was also associated with an increase in T cells and fewer myeloid cells, indicating GLI is an important modulator of the immune response in the pancreas.

Another event related to Hh pathway activation that may contribute to the pathogenesis of chronic pancreatitis includes loss of cilia in epithelial cells. The absence of cilia in pancreatic epithelial cells produces lesions similar to those seen in chronic pancreatitis [66]. Cilia are absent in pancreatic preneoplastic lesions (e.g., PanINs) and human PDAC cells, indicating loss of cilia occurs early during tumor development and is associated with cancer progression [67]. Seeley et al. demonstrated that activation of KRAS, an early event during PDAC initiation, blocks cilia assembly in PanINs and PDAC cells [67]. The authors propose oncogenic KRAS may lead to aberrant activation of the Hh signaling pathway in the absence of cilia. This was supported by the work of Cervantes and colleagues showing that the loss of cilia is associated with overexpression of Hh in the pancreatic epithelium and enhanced PDAC tumorigenesis [68]. Wong et al. suggests cilia may play a dual role in both suppressing and promoting tumorigenesis via the Hh pathway since human basal cell carcinomas are frequently ciliated [69]. The mechanism by which the tumor is initiated may determine the role cilia play in cancer progression. For example, removal of cilia in tumors initiated by Smo mutation inhibited tumor progression, while loss of cilia in tumors induced by GLI mutation increased tumorigenesis [69]. Since cilia are the central location for canonical hedgehog signaling, loss of cilia in tumor epithelial cells may play a role in the switch from canonical to noncanonical activation of the Hh pathway during PDAC tumorigenesis.

Hedgehog Signaling in PDAC Biology

One of the first indications the Hh pathway may be critical in PDAC was the discovery that PanIN lesions express Shh ligand [9, 70, 71]. This is important since components of the Hh pathway, especially the ligands, are undetectable in the normal human pancreas. In support of this notion, Shh overexpression in the developing pancreas of a transgenic mouse is sufficient to initiate PanIN-like precursor lesions [9, 72]. PDAC cells express GLI1 in the absence of ligand, indicating that ligand-independent activation of downstream components of the Hh pathway is occurring [55]. In fact, activation of the Hh pathway is unique in PDAC cells and the stroma, where activation of downstream components of the Hh pathway in the tumor epithelial cells occurs through noncanonical signaling while the surrounding stroma utilizes canonical Hh signaling. Cross talk between the tumor cells and the tumor microenvironment demonstrates a complex interplay that both promote cancer progression and tumor suppression as PDAC advances to the later stages of disease.

Role of Hedgehog Pathway in PDAC Cell Compartment

As mentioned above, activation of the GLI transcription factors in PDAC cells is ligand independent. Nolan-Stevaux et al. showed that deletion of the Smo receptor in pancreatic epithelium did not inhibit GLI expression in these cells, indicating that GLI is activated using a noncanonical mechanism [57]. This activation is mediated through cross talk with several other pathways including KRAS, TGFβ, and EGFR (Fig. 2).

GLI1 expression is a critical component of KRAS-driven PDAC. Using a mouse model with simultaneous activation of oncogenic KRAS and GLI1 in pancreatic epithelial cells, Rajurkar et al. showed that GLI1 promotes KRAS-driven PDAC precursor lesions [8]. Using a similar KRAS model, Mills and colleagues further demonstrated a key role for GLI1 in initiation of PDAC, where loss of GLI in the presence of oncogenic KRAS leads to a significant decrease in PanIN lesions and ablated PDAC formation [11]. Rajurkar et al. suggest the underlying mechanism of this promoting effect was GLI1-dependent regulation of I-kappa-B kinase epsilon (IKBKE), a major regulator of NF-κB pathway in PDAC cells [8], while Mills et al. identified cytokine IL-6 pancreatic fibroblasts as one of the mediators of GLI1-regulated PDAC initiation [11]. IL-6 expression in the stromal compartment induces activation of STAT3 in adjacent epithelial cells, leading to premalignant lesions in PDAC. Transgenic overexpression of GLI2 in the pancreatic epithelium did not induce PanIN lesion development, but a significant percentage of mice developed highly undifferentiated pancreatic tumors [7]. Interestingly, simultaneous overexpression of GLI2 and activation of KRAS cooperate to develop PanIN lesions, indicating interaction between Hh and KRAS plays a role in PDAC initiation [7]. GLI3 has also been shown to play a role in KRAS-regulated oncogenic functions, specifically autophagy. Evidence suggests that autophagy may promote tumor

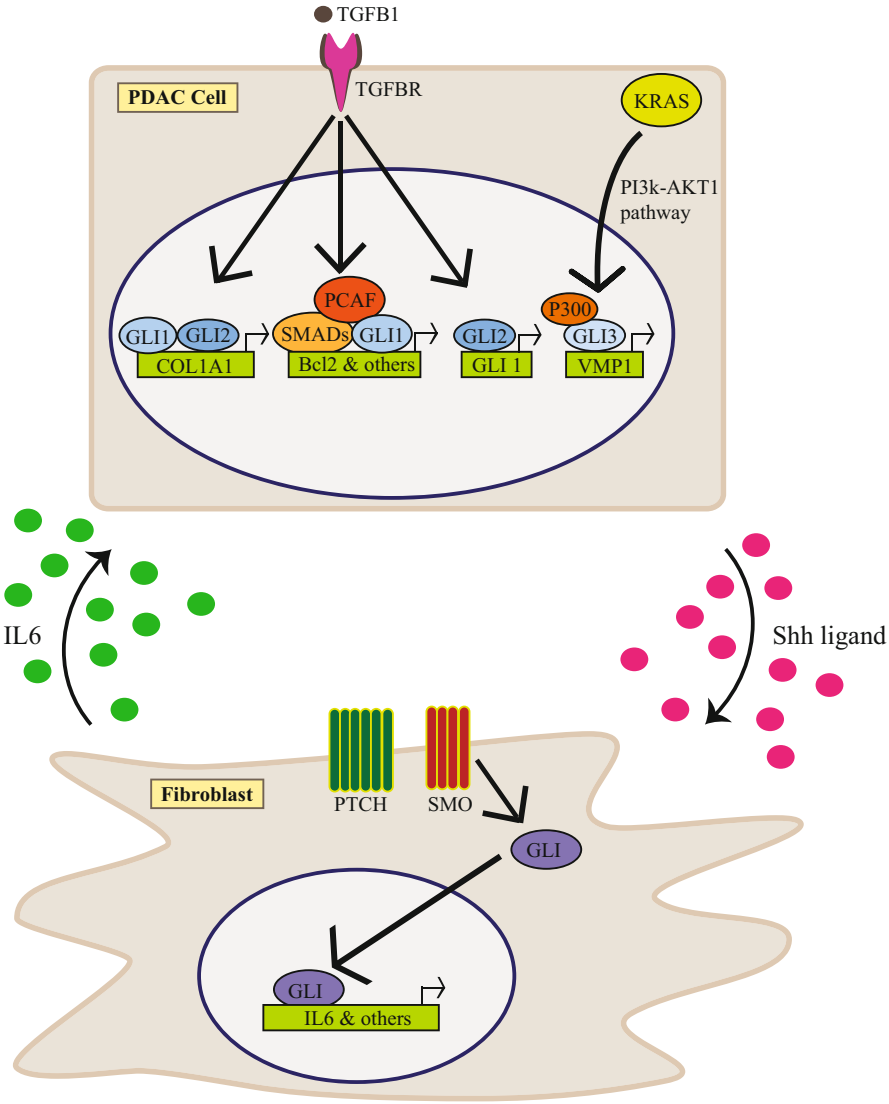

Fig. 2 Paracrine signaling between tumor epithelial cells and fibroblasts. Tumor epithelial cells employ noncanonical activation of GLI. Two mechanisms for ligand-independent activation include KRAS and TGFβ pathways. Oncogenic KRAS activates PI3K-AKT1 pathway to induce binding of GLI3 and p300 to the promoter of VMP1, leading to autophagosome formation. TGFβ induces COL1A1 expression through formation of a transcriptional complex of GLI1 and GLI2 (Martin E. Fernandez-Zapico unpublished observation). TGFβ also induces formation of the transcriptional complex of SMAD2, SMAD4, and PCAF to induce BCL2 transcription. In addition, TGFβ promotes GLI2 binding to GLI1 promoter and induces transcription. Shh ligand expressed by tumor cell initiates Hh signaling in neighboring stromal cells through paracrine signaling. Hh signaling in stromal cells promotes expression of GLI target genes including IL-6 and COL1A1, which stimulate neighboring cancer cells

progression by allowing cancer cells to escape low-nutrient conditions. KRAS-driven tumors display constitutively activated autophagy [73]. KRAS-induced autophagy in PDAC cells lines promotes binding of GLI3 and p300 through the KRAS-PI3K-AKT1 pathway to the promoter of the autophagy gene vacuole membrane protein 1 (VMP1), a pancreatitis-associated protein [74]. In this case GLI3 acts as an activator of transcription, stimulating expression of VMP1 and promoting autophagosome formation. Oncogenic KRAS has also been shown to inhibit autocrine signaling of ligand in PDAC cells, promoting paracrine signaling between the PDAC cells and the stroma [11, 55]. This shift from autocrine to paracrine signaling is through KRAS activation of the downstream effector dual specificity tyrosine phosphorylation-regulated kinase (DYRK1B), which inhibits GLI1 expression through expression of the repressor, GLI3 [75].

Similar to KRAS, transforming growth factor-β (TGFβ) induces ligand-independent activation of GLI proteins in different tumoral compartments including PDAC cells. TGFβ induces GLI2 expression in PDAC cells through Smad3, β-catenin, and LET-dependent upregulation of GLI2 transcription [43, 76]. Further analysis of this phenomenon showed that TGFβ can promote the formation of a new transcriptional complex of GLI1, SMAD2, SMAD4, and the histone acetyltransferase, PCAF, to regulate TGFβ-induced BLC2 gene expression in PDAC cells [77]. Activation of TGFβ is associated with epithelial to mesenchymal transition (EMT), metastasis, and tumor growth [78]. GLI1 seems to antagonize this effect through binding of the E-cadherin (CDH1) promoter [79]. E-cadherin is a transmembrane protein critical for cell adhesion, and lowered expression of E-cadherin leads to increased cell motility. Lowered expression of GLI1 in advanced PDAC was associated with a loss of E-cadherin and promotion of EMT, indicating that GLI expression in the later stages of PDAC may actually inhibit metastasis [79]. Conversely, GLI1 has also been shown to regulate mucin 5AC (MUC5AC) expression in PDAC [80]. Increased MUC5AC expression is associated with migration and invasion of PDAC through GLI1 attenuation of E-cadherin/β-catenin signaling. Inhibition of MUC5AC could potentially restore E-cadherin-mediated cellular adhesion and decrease β-catenin nuclear accumulation, lowering the migratory ability of PDAC cells [80]. This somehow discordant effect could be explained by the different models and experimental conditions used by these research groups [79, 80]. Other molecules controlling cell adhesion have been associated with active Hh signaling, including galectin-1 (Gal1). Gal1, a regulator of cell-cell and cell-ECM adhesion, is highly expressed in PDAC stroma and has been shown to regulate acinar to ductal metaplasia, thought to be a critical step in PDAC initiation where acinar cells take on the pancreatic duct cell phenotype, in part through promotion of Hh signaling [81].

Epidermal growth factor (EGFR) signaling is aberrantly activated in a large number of PDACs, and it has been shown to influence Hh signaling through stimulation of GLI target genes [10]. Eberl et al. identified a set of GLI-regulated genes with enhanced expression in the presence of an active EGFR signal including JUN, SOX9, SOX2, FGF19, and CXCR4 [10]. Both SOX2 and SOX9 are transcription factors involved in regulation of stem cells, indicating that HH-EGFR response

may play a critical role in cancer stem cell (CSC) maintenance though a GLI1-dependent mechanism. A number of groups have also demonstrated that canonical Hh pathway plays a critical role in maintenance of CSCs through regulation of genes associated with stemness [20, 82, 83]. Inhibition of Hh signaling has been shown to interfere with self-renewal of CSCs and may lead to chemosensitivity [84–86]. Gu et al. demonstrated that a combination of radiation with Hh inhibition leads to a significant decrease in EMT in PDAC [87].

Another Smo-independent mechanism for GLI1 activation includes the G-protein subunit, Gα. As previously mentioned, Smo is coupled with a heterotrimeric G-protein complex that activates the Hh intracellular signaling cascade. However, it is unclear if G-protein coupling of Smo occurs in carcinogenesis. Gα has been shown to affect activation of GLI1 independent of Smo [88]. A potential target of Smo-Gα includes the kinase DYRK1A, which acts as a positive regulator of GLI transcription, but may also function as an inhibitor of Hh signaling through indirect inhibition of the GLI transcriptional coactivator MKL1 (MAL) [89]. A MKL1 interactor, Jumonji domain-containing protein 1A (JMJD1A), is a histone demethylase that binds directly to GLI, inhibiting its degradation [89]. Similar to the epigenetic regulator JMJD1, He et al. demonstrated that GLI1 regulates the DNA methyltransferases, DNMT1 and DNMT3a, in PDAC through binding to the DNMT1 gene promoter [90]. GLI1-/DNMT1-mediated methylation may promote invasion and metastasis through activation of oncogenes. Through in vivo RNAi screen for epigenetic regulators regulating PDAC biology, Huang et al. identified BRD2 and BRD3, members of the BET family of chromatin readers, as regulators of PDAC growth in part through modulation of GLI transcription factors [91]. BET bromodomain inhibition led to a decrease in GLI activity in PDAC cells [91]. Huang et al. found the transcriptional activation of GLI1 and GLI2 is mediated through physical interaction with BET proteins. These data suggest the BET proteins may play an important role in regulation of the noncanonical Hh pathway and offers a new strategy for targeting this pathway.

Hedgehog Signaling in PDAC Tumor Microenvironment

As described in some of the chapters of this book, desmoplastic reaction (DR) is a typical feature of PDAC tumor microenvironment (TME). The TME is highly fibrotic and composed of different cell types including pancreatic stellate cells (PSCs), fibroblasts, endothelial cells, nerve cells, immune cells, and acellular components of the extracellular matrix (ECM). Interaction between the stromal cells and malignant epithelial cells appears to play a critical role in tumorigenesis in PDAC. At this point, it is not entirely clear whether the presence of stroma promotes or restrains cancer progression. Initial studies indicated stroma promotes cancer progression, where increased levels of stroma were correlated with a poor prognosis and depletion of stroma improved prognosis [92–94]. However, more recent studies have shown the stroma may actually work to restrain the growth of the tumor, indicating there is still more to learn regarding its role in PDAC [95–97].

Several reports demonstrate that activation of the Hh pathway in the stroma is ligand dependent, and it is usually initiated by signaling from neighboring PDAC cells, leading to an increase in growth factor and chemokine production in stroma, promoting DR in the TME (Fig. 2) [98, 99]. For example, Hh-activated PSCs are thought to play an important role in stromal production in response to pancreatic injury and inflammation [100–102]. PSCs are responsible for production of the many ECM proteins in the TME, including collagen types I, III, and IV and fibronectin [103, 104]. Collagen type I is encoded by two genes, COL1A1 and COL1A2, which are targets of the GLI proteins in PSCs and fibroblasts (Martin E. Fernandez-Zapico unpublished observation). As a result of the dense DR in PDAC, the TME is hypovascular and hypoxic [105, 106]. Hypoxia is associated with worse clinical outcome due to an increase in tumor growth rate and metastasis [107]. Tumor hypoxia leads to activation of hypoxia-inducible factor 1α (HIF-1α), a transcription factor that is overexpressed in PDAC and is associated with increased EMT and invasion [108, 109]. HIF-1α induces noncanonical activation of GLI1 in pancreatic cancer cells to promote Snail expression and EMT [109]. These studies show the critical role the TME plays in tumor progression through promotion of invasion and metastasis of PDAC.

As previously mentioned, chronic inflammation is a known risk factor for PDAC. Inflammatory cells are an important part of the stromal reaction associated with PDAC and may play a role in disease progression through upregulation of several pathways including Hh [110]. IL-6 and other proinflammatory markers are elevated in PDAC patients [111, 112]. As previously mentioned, IL-6, a target of GLI1, induces STAT3 activation in PDAC cells [11, 112], a transcription factor essential for the formation of PanIN lesions and their progression into tumors [113–115]. This indicates that Hh mediates the communication between the stroma and epithelial cells playing a critical role in tumor initiation.

Based on the tumor-promoting features of the stroma, Hh inhibition appears to be a promising target in PDAC treatment. In support of this theory, Olive et al. found that depletion of the stroma by co-administering the Hh inhibitor, IPI-926, with gemcitabine, led to disease stabilization through more efficient delivery of the drug to the tumor [93]. This successful result led to clinical trials with the Hh inhibitor, vismodegib (GDC-0449), for treating PDAC [116, 117]. Unfortunately, these studies showed patients treated with an Hh inhibitor either showed no improvement or had a higher rate of disease progression than placebo.

These failed clinical trials bring into question the prevailing paradigm that the stroma supports tumorigenesis in PDAC. In fact, more recent studies have shown the stroma may actually work to restrain the growth of the tumor [95–97]. Rhim et al. demonstrated that loss of stroma using an Hh inhibitor or genetic ablation of Shh leads to more aggressive tumors [95]. Also, a preclinical study found that Hh inhibition at the PanIN stage led to increased tumor progression, while Hh activation in advanced stages of PDAC slows down tumorigenesis [96]. Similarly Mills et al. showed loss of GLI1 in a PDAC model with activated KRAS, and concomitant loss of p53 led to accelerated progression [118]. In this study the authors suggested a significant decrease in expression of FAS/FASL as a potential

underlying mechanism for increased tumorigenesis in animals lacking GLI1. Decreased expression of FAS/FASL is associated with lower apoptosis and may lead to increased tumor progression. In addition, lowered expression of GLI1 in advanced PDAC was associated with a loss of E-cadherin and promotion of EMT [79]. These unexpected findings reveal an incomplete understanding of the role of the Hh pathway in all stages of PDAC. While Hh inhibition appears to be a potential target for PDAC treatment, it seems that increased Hh expression may actually have a tumor repressive effect in the latter stages of PDAC. A study by Fendrich et al. showed that Hh signaling promotes acinar differentiation, indicating increased GLI levels may actually promote differentiation and slow tumorigenesis [59].

One important caveat is that Hh inhibition in patients is unlikely to completely inactivate Hh signaling. As previously mentioned, Hh coreceptors Gas1, Boc, and Cdo bind Hh ligand and enhance Hh pathway signaling. These coreceptors are upregulated in PDAC and are required for Hh signal transduction [119]. Surprisingly, deletion of two coreceptors led to a more potent tumor compared to wild type. Deletion of all three receptors resulted in lower angiogenesis and decreased tumorigenesis, indicating HH signaling effects in PDAC are dose dependent [119]. These findings may explain the failure of Hh inhibitor clinical trials, where partial inhibition may actually increase tumorigenicity through increased angiogenesis.

Targeting Hedgehog Pathway in PDAC

As mentioned above, thus far, Smo inhibition of Hh pathway in PDAC has had disappointing results. One reason for this poor response may be that PDAC cells utilize Smo-independent activation of the Hh pathway. In addition, SMO is critical to several cellular functions, and loss of SMO could be deleterious to cells independent of Hh pathway activity. Finally, the stage of the PDAC plays a major role in the outcome of Hh inhibition. Based on this data, researchers are looking to target downstream components of Smo signaling, such as GLI inhibitors, as well as utilizing a more individualized approach when selecting a therapy that will work best for each patient. GLI inhibition would be a promising target since it would affect both the tumor and stromal cells. A small molecular inhibitor of GLI1 and GLI2, the Gli-*ANT*agonist (GANT61), acts in the nucleus to block GLI1- and GLI2-mediatied transcription [120]. GANT61 was discovered using a luciferase assay-based screen in HEK293 cells. Studies in mice showed that GANT61 induces strong tumor regression (120), may inhibit pancreatic CSC growth [121, 122], and induces autophagy, contributing to reduced viability [123]. While GANT61 is a potent GLI1 and GLI2 inhibitor, there are no clinical trials currently ongoing for this drug for any type of cancer. Arsenic trioxide (ATO), a popular chemotherapeutic agent, is FDA-approved for the treatment of acute promyelocytic leukemia (APL) where it degrades the PML-RAR fusion protein that drives the disease [124]. Arsenic can inhibit the Hh pathway through inhibition of GLI proteins by directly binding the

GLI zinc finger domain and blocking ciliary accumulation of GLI2 [125, 126]. This allows Hh pathway inhibition downstream of Smo. Combination of ATO and parthenolide (PTL), an herbal medicine, was shown to inhibit growth of pancreatic cancer cells through induction of apoptosis [127]. This study indicates ATO may be a promising target for GLI inhibition, but additional preclinical studies are needed to determine its effectiveness. In another study, Damhofer and colleagues investigated the potency of paracrine signaling of tumor cells in the neighboring stroma [128]. While Hh ligands are highly membrane-associated, they can only target cells diameters away. By inhibiting the release of Hh ligands from PDAC cells, Damhofer et al. found this increased the signaling range of the ligand to adjacent stromal cells. This indicates that endogenous Hh on the cancer cell surface increases signaling range and potency. Based on this theory, Hh-blocking antibodies may lower this activation and decrease the signaling range of PDAC tumors, which may have a different effect than complete ablation of the pathway.

In support of the above statement, Rihm et al. discovered that Shh-deficient tumors were more poorly differentiated, exhibited increased vascularity, and were more aggressive [95]. These results suggest inhibition of the HH/GLI1 axis may have a proangiogenic effect on the tumor. Shh-deficient mice showed increased tumor vasculature, leading the authors to investigate the effect of angiogenesis inhibition through administration of anti-VEGF receptor to tumor-bearing SHH-deficient mice [95]. Anti-VEGF receptor therapy led to a significant improvement in the overall survival of mice-bearing undifferentiated tumors. This indicates that the subset of PDAC patients with undifferentiated tumors with low levels of Hh activity may benefit from some form of anti-angiogenic therapy.

Some antifungal inhibitors have unexpectedly shown promise in PDAC treatment. A screen of FDA-approved drugs identified itraconazole (ITZ) as an Hh inhibitor, most likely through inhibition of Smo, but is distinct from other Smo antagonists [129]. ITZ is commonly administered orally for treatment of a broad range of fungal infections. In addition to inhibition of the Hh pathway, ITZ also inhibits angiogenesis and induces autophagy [130–132]. One case involving a patient with unresectable stage III PDAC showed a positive response to ITZ treatment [133]. Following ITZ treatment for 9 months, the patient's PDAC regressed and was able to be treated surgically. This makes ITZ an interesting candidate for PDAC treatment.

As an alternative to direct Hh inhibition, some chemopreventive "natural agents" (nutraceuticals) may also target PDAC CSCs through inhibition of self-renewal and early metastasis [85]. Consumption of fruits and vegetables is strongly correlated with a lower PDAC incidence [134]. These natural compounds prevent cancer through inhibition of multiple signaling pathways [135, 136]. For example, sulforaphane, found in cruciferous vegetables, has been found to inhibit self-renewal of CSCs through inhibition of downstream components of the Hh pathway, Nanog, and Oct-4, which are pluripotency maintaining factors [137]. In addition to sulforaphane, other nutraceuticals that inhibit the Hh pathway include epigallocatechin-3-gallate (EGCG), found in green tea polyphenols, and the

flavonoid, quercetin [85]. EGCG inhibits CSCs through inhibition of pluripotency transcription factors (Nanog and Oct-4), EMT markers (twist-1 and Zeb-1), and components of the Hh pathway. Quercetin shows a synergistic inhibitory effect on GLI and TCF/LEF transcriptional activity in CSCs when used in combination with EGCG [85].

Conclusion

The Hh pathway is highly complex and its mode of action is cell type dependent in PDAC. This complexity has made targeting the Hh pathway for treatment of PDAC difficult. While it is clear that the stroma contributes significantly to PDAC tumorigenesis, it is not known which components of the stroma are tumorigenic and which repress tumor growth. Delineating the role of each cell type in the stroma in PDAC will be critical when targeting stromal depletion as a drug therapy. In addition, the Hh pathway plays multiple roles in carcinogenesis by its involvement in both the cancer cells and stroma, where it contributes to tumor initiation and progression in early carcinogenesis but may switch to a tumor suppressor as the cancer progresses. This multifunctionality makes it critical to understand the role of the Hh pathway in all stages of cancer, particularly in the advanced stage when the cancer is most often detected. In addition, development of Hh inhibitors downstream of SMO offers promise as an effective treatment for advanced PDAC since this treatment would target both the cancer cells and the stroma.

While SMO inhibition was not successful for patients with advanced metastatic PDAC, there is some indication that this treatment may be beneficial before cancer develops, such as during chronic pancreatitis. As mentioned in this chapter, the Hh pathway is aberrantly activated in pancreatitis and preneoplastic lesions, and activation of this pathway during inflammation may drive the pancreas toward tumor formation. GLI acts a tumor promoter in early tumorigenesis, indicating that inhibition of GLI may block tumor initiation. Therefore, Smo inhibition in patients with a risk of developing PDAC may be a beneficial chemopreventive in both the early stages of inflammation and during tumor initiation when the Hh pathway is tumorigenic.

Finally, another area of research that should be further investigated is the mechanisms responsible for the transcriptional activation of GLI. Identification of coactivators of GLI may help researchers further elucidate GLI transcriptional regulation and identify new therapeutic targets for PDAC treatment. One potential coactivator of GLI includes the Zic family of proteins [138]. Similar to GLI, Zic proteins contain a zinc finger-binding domain. In addition, Zic and GLI proteins have a nearly identical binding sequence, although Zic binds GLI promoters with a lower binding affinity [138]. Zic proteins have been shown to interact with GLI through their DNA-binding domains to synergistically enhance gene expression, indicating Zic may act as a transcriptional coactivator of GLI [138, 139]. Further clarification of coactivators, such as Zic proteins, in GLI transcriptional activation may serve as a potential avenue to interrupt GLI signaling in PDAC.

Cross-References

- ▶ Animal Modeling of Pancreatitis-to-Cancer Progression
- ▶ Apoptosis: Signaling Pathways in Pancreatic Cancer Pathogenesis
- ▶ Cell Cycle Machinery and Its Alterations in Pancreatic Cancer
- ▶ Developmental Molecular Biology of the Pancreas
- ▶ EGFR (ErbB) Signaling Pathways in Pancreatic Cancer Pathogenesis
- ▶ Emerging Therapeutic Targets in Pancreatic Adenocarcinoma
- ▶ Epidemiology and Prospects for Prevention of Pancreatic Cancer
- ▶ Epigenetics and Its Applications to the Progression Model of Pancreatic Cancer
- ▶ Molecular Pathology of Pancreatic Endocrine Tumors
- ▶ Mouse Models of Pancreatic Exocrine Cancer
- ▶ Notch Signaling in Pancreatic Morphogenesis and Pancreatic Cancer Pathogenesis
- ▶ Pancreatic Cancer Stem Cells
- ▶ Pathologic Classification and Biological Behavior of Pancreatic Neoplasia
- ▶ Role of Tumor-Stromal Interactions in Pancreatic Cancer Invasion and Metastases
- ▶ Smad4-TGF-β Signaling Pathways in Pancreatic Cancer Pathogenesis
- ▶ The Molecular Pathology of Precursor Lesions of Pancreatic Cancer

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Smad4-TGF-β Signaling Pathways in Pancreatic Cancer Pathogenesis

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Contents

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is a deadly cancer with a 9% 5-year survival rate. For reasons that are not readily evident, *KRAS* is mutated in 90–95% of PDAC cases, and this truncal alteration is associated with a high frequency of mutations in crucially important tumor suppressor genes, most notably *CDKN2A* (~90%), a gene that encodes p16, *TP53* (~70%), and *SMAD4* (~50%). Concomitantly, there is overexpression of transforming growth factor beta (TGF-β) isoforms and of high-affinity tyrosine kinase receptors (TKRs) and their ligands. Enhanced cancer cell proliferation and migration mediated by TKRs, combined with loss of beneficial TGF-β-dependent pathways required to restrain uncontrolled cell proliferation, contributes to PDAC's biological aggressiveness. This chapter provides an overview of these issues and focuses on the role of alterations in Smad4 expression and function and aberrant TGF-β signaling that combine to promote pancreatic cancer growth through cell autonomous and paracrine actions, thereby contributing in an important manner to PDAC pathobiology.

Keywords

Smad4 · Smad7 · TGF-β · Canonical signaling · Non-canonical signaling · Tumor microenvironment · Pancreatic cancer · Angiogenesis · TGF-β

Pancreatic Ductal Adenocarcinoma

Disease Description

Pancreatic ductal adenocarcinoma (PDAC) is the third leading cause of cancer mortality in the United States, with a 5-year survival of 8–9% [1]. Due to the increasing incidence of both type 2 diabetes mellitus and obesity as well as the aging of the population, all of which are recognized risk factors for PDAC [1–4], it is predicted that the incidence of PDAC will continue to increase in the United States. Moreover, improving survival rates in other cancers, such as breast cancer, will accentuate PDAC's impact as a therapy-recalcitrant cancer, and it is anticipated that

by 2020 or a few years beyond 2020 PDAC will become the second leading cause of cancer death in the United States [5].

Overview of Cardinal Features of PDAC

Cardinal features of PDAC include a propensity to be locally invasive as well as metastatic, and to exhibit resistance to chemotherapy or radiotherapy [6, 7]. Approximately 20% of patients with PDAC have a resectable cancer at clinical presentation. This low percentage of patients who are candidates for surgery is due to advanced stage at presentation in most cases, and the absence of biomarkers for early detection. As shown in Table 1, PDAC's biological aggressiveness is likely due to the presence of several high-frequency major driver mutations that include *KRAS* (90–95% mutation rate), *CDKN2A* (~90%), *TP53* (~70%), and *SMAD4* (~50%) in combination with many low-frequency driver mutations that add complexity to the altered genomic landscape and may interfere with attempts at targeted therapies [8]. Additionally, there is constitutive activation of pro-survival pathways including AKT, STAT3, and NFkB that combine to contribute to marked apoptosis resistance, excessive production of tyrosine kinase receptors (TKRs) and their ligands such as transforming growth factor alpha (TGF-α), fibroblast growth factors (FGFs), insulin-like growth factor 1 (IGF-1),

Table 1 Major driver mutations and targetable mutations in PDAC. Gene alteration frequency (%) function

Gene	Alteration	Frequency	Consequence
KRAS	Activating mutations	~92%	Mitogenic signaling that contributes to PDAC initiation, progression, and metastasis
CDKN2A	Inactivating mutations	~90%; ~10% is epigenetically silenced	Loss of ability to suppress cell cycle progression, causing enhanced proliferation
TP53	Inactivating mutations	~70%	Apoptosis resistance, chemoresistance, increased metastasis
SMAD4	Homozygous deletions or missense mutations	~24% deleted ~14% mutated ~6% multiple alterations	Perturbations in canonical TGF-β signaling
BRCA1	Amplification, mutation, and deletion	~5% amplified ~1% mutated ~1% deleted	Genomic instability due to loss of DNA damage repair capacity and inability to activate checkpoint mechanisms
BRCA2	Mutation and amplification	~4%mutated ~2% amplified	Loss of ability to perform homologous recombination
PALB2	Mutation and amplification	~4%mutated ~1% amplified	Loss of ability to perform homologous recombination

Data for frequency of gene alterations for *SMAD4*, *BRCA1*, *BRCA2*, and *PALB2* are from TCGA and cBioportal. Mutations in *BRCA1*, *BRCA2*, and *PALB2* define a subgroup of PDAC patients that can have a marked therapeutic response to platinum agents [17] and poly(ADP-ribose) polymerase (PARP) inhibitors

and hepatocyte growth factor (HGF), a dense stroma that impedes drug delivery, and suppression of cancer-directed immune mechanisms [6–10].

PDAC exhibits many features of the hallmarks of cancer, including self-sufficiency in growth signals, insensitivity to growth inhibitory pathways such as those that are usually activated by transforming growth factor beta (TGF-β), immune evasion, and a capacity to invade and metastasize [11]. These aberrant processes have been attributed to ~70 genetic alterations impacting many signaling pathways [12]. Subsequent deep whole genome sequencing and copy number variation studies suggest that PDAC has additional mutations in numerous genes, such as *ROBO2*, ARID2, *SLIT2*, *MAP2K4*, and *ATM*, and that in some PDACs there are deletions, rearrangements, and amplifications of large fragments of DNA and small regions of hypermutation termed kataegis [13, 14], underscoring the complex and heterogeneous nature of PDAC.

The TGF-β Superfamily of Ligands

TGF-β Superfamily

The TGF-β superfamily consists of growth factors that are expressed in all vertebrates, including humans, rodents, and *Xenopus*, and that has been subdivided into two main branches on the basis of sequence homologies [15]. Thus, one branch includes TGF-β isoforms, activins, and nodal, whereas the other branch includes such growth factors as muellerian inhibitory substance (MIS), growth and differentiation factors (GDFs), and bone morphogenetic proteins (BMPs) which has numerous members [15, 16]. The three mammalian TGF-β isoforms share ~70–80% amino acid sequence homology and are synthesized as precursors that are cleaved into biologically active 25 kDa dimers [17]. Differences in biological actions are dictated by temporal and spatial regulation of expression [18]. In general, TGF-βs enhance the proliferation of mesenchymal cells and inhibit the proliferation of epithelial cells. However, TGF-βs are multifunctional and can exert numerous additional biological actions in a context-dependent manner, such as induce differentiation and apoptosis, modulate the expression of integrins, alter extracellular matrix deposition, and direct the traffic of inflammatory cells [15–18].

Canonical TGF-β Signaling

As depicted in Fig. 1, TGF-β actions are initiated following ligand binding the type II TGF-β receptor (TβRII) homodimer [19, 20]. TGF-β itself also binds as a homodimer, and the resulting complex associates with a type I TGF-β receptor (TβRI) homodimer that is thereby phosphorylated within a SGSGSG sequence and activated as a serine-threonine kinase [19, 20]. Activated TβRI phosphorylates Smad2 and Smad3 but not Smad4. That is because phosphorylation occurs on the last two serine amino acids of the C-terminal SSXS residues of Smad2 and Smad3, located in their mad homology

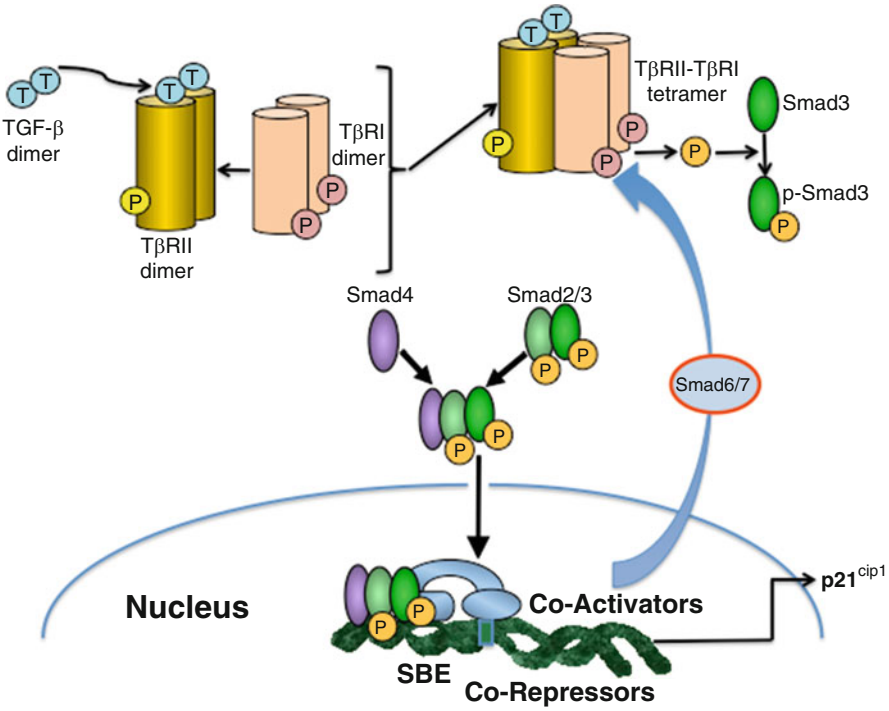

Fig. 1 Canonical TGF-β signaling. Following binding of a TGF-β dimer to the TβRII dimer, the TβRI dimer is recruited to form a heterotramer with TβRII, and the kinase activity of TβRI is thereby activated. The serine-threonine kinase activity of TβRI in turn leads to the phosphorylation of Smad2 and Smad3. The figure depicts the phosphorylation of Smad3. These receptor-activated phospho-Smads (p-Smad2 and p-Smad3) form complexes with Smad4 and translocate to the nucleus where they associated with coactivators and corepressors to interact with specific Smad-binding elements (*SBE*) in the DNA. In the example shown, interaction with coactivators leads to the induction of $p21^{cip1}$. In addition, inhibitory Smad6 and Smad7 are induced by TGF-β, leading to negative feedback effects on Smad2/3 phosphorylation and actions as explained in the text

2 (MH2) domain, and this motif is absent in Smad4 [19–21]. In addition, the Smad anchor for receptor activation (SARA) restrains Smad2 and Smad3 near the cell membrane, thereby enhancing TβRI's ability to access Smad2/3 to [22]. Once phosphorylated, Smad2 and Smad3 oligomerize with Smad4 and the resulting complexes translocate to the nucleus to regulate gene transcription in conjunction with corepressors and coactivators [19–23]. In general, activins also act by inducing the phosphorylation of Smad2/3 whereas BMP signaling induces phosphorylation of Smad1, 5, and 8 [24]; in both pathways receptor-activated Smads associate with Smad4.

Smad Cytoplasmic-Nuclear Shuttling

Smad2 and Smad3 are endowed with an intrinsic capacity to keep shuttling between the cytoplasm and nucleus [25]. However, under basal condition, Smad2 and Smad3

are mostly localized in the cytoplasm, in part due to SARA's ability to tether both ligand-activated transcription factors near the cell membrane and to mask the nuclear import signal in the MH2 domain [22]. This domain also mediates Smad2/3 oligomerization, transcriptional activity, and protein-protein interactions [19–21]. By contrast, the amino-terminal MH1 domain facilitates Smad2/3 binding to DNA [21].

Smad Nuclear Retention

Smad2/3 transcriptional regulatory actions in the nucleus are dependent on nuclear retention of these complexes. Although Smad4 is not required for retention of Smad complexes in the nucleus, it contributes in an important manner to the formation of active transcriptional complexes [21]. Thus, following nuclear translocation, Smad4 binds to a CAGAC motif and Smad-interacting DNA-binding proteins that function as coactivators or corepressors, and that include such proteins as p300/CBP, AP-1, FAST-1, Milk, and OAZ, enabling the Smad complexes to modulate gene transcription [26, 27]. Moreover, recent studies indicate that Smad transcriptional actions are modulated by cell-specific transcription factors and chromatin machinery, and by transcription factors activated by cross-interacting signaling pathways, all of which combine to markedly refine gene expression output [27].

The above complex regulatory actions require the presence of Smad2/3 complexes and Smad4 in the nucleus, and the nuclear localization of these Smads is also finely orchestrated to allow for effective modulation of gene expression. For example, TGF-β signals to attenuate Smad2 exit from the nucleus [28], thereby promoting the nuclear retention of p-Smad2. Conversely, nuclear phospho-Smad2/3 can be dephosphorylated by phosphatases and once stripped of their phosphate, Smad2/3 moieties shuttle out of the nucleus and into the cytoplasm aided by Ran-binding protein 3 (RanBP3), a Ran-binding protein that resides in the nucleus [29]. Ran is a ras-related nuclear protein that interacts with regulator of chromosome condensation 1 (RCC1) and binds GTP [29]. RanBP3 associates with Ran, nuclear pore proteins, RCC1, and Exportin 1 that is also known as Chromosomal Maintenance 1, or CRM1. Consequently, RanBP3 is able to drive dephosphorylated Smad2/3 from the nucleus, through the nuclear pores, and into the cytoplasm [29], thereby inhibiting Smad2/3 transcriptional actions. By contrast, Smad4 contains a nuclear export signal that impedes nuclear localization when TβRI is inactive, and Smad4 exit from the nucleus into the cytoplasm is directly mediated by CRM1 [30].

TGF-β-Mediated Autoinhibition

TGF-β signaling cascades eventually lead to the activation of autoinhibitory pathways. Thus, TGF-β induces the expression of two inhibitory Smads, Smad6 and Smad7, that are components of a negative feedback loop that deactivates TGF-β signaling [31]. Inhibitory Smads bind to the activated TβRI and inhibit the phosphorylation of Smad2/3 [31]. In addition, Smad7 recruits Smurf1 (Smad-

ubiquitilation regulatory factor 1 (Smur1) and Smurf2 to the receptor complex, leading to the ubiquitilation and degradation of TβRI [32, 33]. Ubiquitilation can be reversed through the actions of the USP15 deubiquitilation enzyme, which prevents TβRI degradation and promotes continued signaling [34]. Conversely, Smad7 associating proteins, including STRAP, GADD34/PP1c, and the Yes-Associated Protein 65 (YAP65), enhance the inhibitory actions of Smad7 to attenuate TGF-β signaling through a variety of mechanisms [35]. For example, PP1c is the catalytic subunit of protein phosphatase 1, and it inhibits TGF-β signaling by dephosphorylating TβRI [35].

Modulation of TGF-β Actions

TGF-β actions can be modulated through additional mechanisms, underscoring the importance of negative feedback loops in the regulation of this important pathway. For example, the BMP and activin membrane-bound inhibitor (BAMBI) is a negative regulator of TGF-β/BMP/activin signaling [36]. BAMBI disrupts TGF-β and BMP effects on transcription, inhibits TGF-β action on Smad2/3 phosphorylation, and antagonizes TGF-β's antiproliferative actions [36]. BAMBI acts by interfering with formation of the TβRII-TβRI heterotetramer, and by associating with Smad7 and TβRI to abrogate TβRI-Smad3 interaction, thereby specifically blocking Smad3-mediated effects [36]. Another example of negative feedback regulation is represented by the actions of SnoN and c-Ski, each of which contains a Smad4-binding domain and both SnoN and c-Ski are able to interact with Smad4 as well as Smads2/3, thereby preventing Smad complexes from activating gene transcription [37]. A third example is represented by Smad3's propensity to undergo ADP-ribosylation as a result of the actions of poly (ADP-ribose) polymerase-1 (PARP-1), which results in Smad complex separation from DNA and decreased transcriptional responses [38].

Suppression of TGF-β-Mediated Autoinhibition

Pathways that inhibit TGF-β actions are also negatively regulated, assuring a fine-tuning of the spectrum of downstream signaling cascades. Thus, Smad7, c-Ski, and SnoN are all negatively regulated by the RING-type E3 ubiquitin ligase Arkadia that is encoded by the *RNF111* gene [37]. Arkadia induces ubiquitin-dependent degradation of Smad7, c-Ski, and SnoN [37]. Moreover, transcription can be enhanced when Smad2/3 become acetylated by TGF-β-induced association with CBP/p300 [39].

There are also indirect mechanisms to interfere with TGF-β-mediated autoinhibition. One interesting example is the ability of activated TβRI to become covalently linked to the SUMO polypeptide, a process called sumoylation [40]. Once sumoylated, TβRI-mediated activation of Smad2/Smad3 is enhanced as a consequence of an improved association of the Smad complex to the receptor

[40]. Thus, sumoylation of TβRI leads to enhanced transcriptional activity by TGF-β.

TGF-β Actions in the Normal Pancreas

The normal pancreas expresses wild-type Smad4 and its various cell types are presumed to be able to respond to TGF-β present in the circulation in a physiological manner. In addition, there are low levels of TGF-β that are synthesized and expressed in the exocrine and endocrine cells of the pancreas [41]. By immunostaining, all three TGF-β isoforms are more abundant in the endocrine islet cells than in either the acinar or ductal cells [42]. The potential physiological importance of TGF-βs in the normal pancreas is evidenced by the observation that mice expressing a dominant-negative form of TβRII (to attenuate TGF-β signaling) in the pancreas exhibit increased acinar cell proliferation and decreased differentiation [42], indicating that TGF-β restrains mitogenesis and promotes differentiation in acinar cells. Interestingly, activation of the pancreatic cholecystokinin (CCK) receptor by caerulein in mice expressing a dominant-negative form of TβRII is associated with a decreased inflammatory response when compared with wild type mice [43]. Thus, some components of TGF-β signaling may modulate immune events in the pancreas and may contribute to caerulein-induced pancreatitis.

TGF-β and Pancreatic Cancer

Loss of TGF-β-Mediated Growth Inhibition in Cancer

In many cell types where TGF-β inhibits proliferation, TGF-β suppresses the G1 phase of the cell cycle by upregulating cyclin-dependent kinase (CDK) inhibitors such as $p21^{Cip1}$, $p15^{Ink4b}$, $p27^{Kip1}$, and p16 and by downregulating drivers of the cell cycle, including Cdc25A, CDK2/CDK4, cyclin A, cyclin E, and p34cdc2 [44]. In culture, PCCs exhibit attenuated growth-inhibitory responses to TGF-β or altogether fail to be growth inhibited [45] in standard two-dimensional cell cultures.

In general, loss of TGF-β-mediated growth inhibition may be due to decreased expression or mutation of TβRII or TβRI. Mutations within relatively unique repeat sequences in TβRII may occur in cancers that exhibit defective mismatch repair and microsatellite instability; these mutations occur within an adenine mononucleotide repeat human TβRII cDNA, due to deletions or insertions of adenines at nucleotides 709–718. For example, mutations in the *TGFBR2* gene may occur in colorectal cancers (CRCs) as microsatellite instability [46]. However, CRCs may also harbor *TGFBR2* mutations in microsatellite stable colorectal cancers [47]. In addition, expression of TβRII may be lost due to other mechanisms such as mutations in the *TGFBR2* gene promoter. In PDAC, it was previously reported that *TGFBR1* and *TGFBR2* are only mutated in ~1% and ~4% of PDAC cases, respectively

[48]. Moreover, three of four of the *TGFBR2* mutations were of the type associated with microsatellite instability [48].

Lessons from The Cancer Genome Atlas

SMAD4, originally identified as a gene deleted in pancreatic carcinoma locus 4 (*DPC4*) on chromosome 18q [49], exhibits allelic loss in ~90% of PDACs, with homozygous deletion occurring in ~30%. Moreover, analysis of this gene has revealed the presence of inactivating mutations in ~20% of PDACs, most commonly occurring within the DNA binding MH1 domain or transcriptional activation MH2 domain. In addition, in some PDACs, there are frame-shift and nonsense mutations in the *SMAD4* gene that result in loss of Smad4 function, as well as missense mutations within the MH2 domain that markedly attenuate Smad dimerization efficiency and may lead to rapid ubiquitilation and degradation of the protein [50]. Consequently, cells harboring such alterations are afflicted with multiple functional perturbations, including dysregulated TGF-β signaling, transcription, and metabolism and adhesion.

As shown in Table 2, in addition to the above well-established alterations, recent advances in sequencing technology have yielded new information in regard to gene mutations in PDAC. Thus, results available in The Cancer Genome Atlas (TCGA) and other sites that can be readily searched on cBioportal (www.cbioportal.org/) indicate that there is a 6% overall alteration rate in the *TGFBR1* gene with 6 of nearly 150 PDACs harboring putative passenger mutations and three harboring deep deletions. Moreover, as indicated in cBioportal, there is an 8% overall alteration rate in the *TGFBR2* gene with two deep deletions, three truncating mutations, four putative passenger mutations, and one putative missense driver mutation. Additionally, the *SMAD2* gene is deleted in 19% of PDACs and mutated in 1% of PDACs, whereas *SMAD3* is amplified in 4% and mutated in 3% of PDACs, *SMAD6* is amplified in 5% and mutated in 1% of PDACs, and *SMAD7* is deleted in 18% and amplified in 1% of PDACs.

As expected based on published data on Smad4 mutations [49, 50], cBioportal indicates that *SMAD4* is deleted in 24% of PDACs, mutated in 14%, and harbors multiple alterations in 6% of PDACs. Deletions, amplifications, or mutations at 1 to 3% frequency range are also observed with BMPR1A, BMPR1B, and BMPR2 that transmit bone morphogenetic protein (BMP) signals, and ACVR2A and ACVR2B that mediate activin signals. Together, these mutations represent an important component of the spectrum of perturbations in TGF-β pathways that contribute to PDAC pathobiology [12] and that include Smad6 or Smad7 overexpression [51, 52], and retinoblastoma protein (pRb) dysfunction [53], which combine with other mechanisms to convert TGF-β from a tumor suppressor to a PCC mitogen [54, 55].

Smad4 and MicroRNAs

MicroRNAs (miRNAs) are short noncoding RNAs, generally 18–25 nucleotides long, that regulate numerous cell functions by targeting specific mRNAs for

Table 2 Mutations in PDAC directly affecting TGF-β signaling components

Gene	Alteration	Frequency	Consequences
SMAD2	Mutation and deletion	~19% mutated ~1% deleted	Perturbations in canonical TGF-β signaling
SMAD3	Amplification and mutation	~4% amplified ~3% mutated	Perturbations in canonical TGF-β signaling
SMAD6	Amplification and mutation	~5% amplified ~1% mutated	Perturbations in negative feedback regulation
SMAD7	Amplification and mutation	~18% deleted ~1% amplified	Perturbations in negative feedback regulation
TGFBR1	Mutation and deletion	~4% mutated ~2% deleted	Loss of negative growth constraints on proliferation
TGFBR2	Mutation, deletion, and amplification	~5% mutated ~1% deleted ~1% amplified	Loss of negative growth constraints on proliferation
TGFBR3	Deletion and amplification	~4% deleted ~2% amplified	Loss of negative growth constraints on proliferation

The high frequency of *SMAD2 mutations* and *SMAD7* deletions underscores the important role of aberrant Smad signaling in PDAC pathobiology

degradation and/or translational repression [56, 57]. Multiple miRNAs have been implicated in PDAC pathobiology based on their altered expression in PDAC and evidence for biological actions, and several miRNAs are known to exert their effects by acting on Smad4. For example, miRNA-182-5p targets Smad4 and RECK in human bladder cancer [58], miRNA-199a attenuates canonical TGF-β signaling by targeting Smad4 in gastric cancer cells [59], and miRNA-224 enhances proliferation of hepatocellular carcinoma cells [60]. In PDAC, Smad4 is targeted by miRNA-421, miRNA-483-3p, and miRNA-301a-3p [61–63]. Conversely, loss of Smad4 in PDAC is associated with decreased miR-494 expression, allowing for FOXM1 to be upregulated and thus leading to increased nuclear translocation of β-catenin, enhanced PCC proliferation and invasion, and decreased response to gemcitabine [64].

TGF-β can also induce the expression of miRNAs and act by exerting posttranscriptional effects. For example, TGF-β is known to induce miRNA-21 expression through a Smad3-Smad4 pathway [65]. In addition, TGF-β enhances miRNA-21 expression in a Smad4-independent manner by promoting pri-miRNA-21 processing into pre-miRNA-21, which is then converted to mature miRNA-21 [66]. Importantly, PDACs are known to overexpress miRNA-21 [67], and this miRNA has oncogenic properties and is therefore considered as an oncomir [68]. In this context, TGF-β's ability to increase miRNA-21 levels in a Smad4-independent manner may enhance its capacity to act as a tumor promoter in a cell-autonomous manner even when Smad4 is mutated or deleted.

Smad4 and Polysomes and Long-Noncoding RNAs

TGF-β has also been shown to increase polysome formation, induce a global increase in translation by activating mTOR, modulate the distribution of mRNA moieties in the cytoplasm and nucleus, and dictate their distribution into polysosmes [69], and these effects are Smad4-dependent. Moreover, long noncoding RNAs (LncRNAs), which are longer than 200 nucleotides and do not encode proteins, have also been implicated in cancer in general and TGF-β actions [70]. Thus, lncRNA-Activated by TGF-β (lncRNA-ATB) is induced by TGF-β in a Smad4-independent manner and sequesters miRNA-200 family members, leading to the upregulation of ZEB1 and ZEB2 and induction of epithelial-mesenchymal transition (EMT) and enhanced metastasis [71]. Together, these actions by TGF-β underscore its ability to exert diverse effects by modulating the expression and function of coding as well as noncoding RNAs.

Smad4 and Mouse Models

Studies with mouse models have revealed that *Smad4* heterozygote mice are viable but develop gastric polyps and eventually invasive gastric cancer in the antrum [72]. By contrast, mutant mice with a homozygous deletion of *Smad4* die by fetal day 7.5 with an abnormal visceral endoderm [73], underscoring the importance of Smad4 for visceral endoderm differentiation. Moreover, rescue experiments have yielded embryos with anterior truncations, indicating that Smad4 regulates anterior patterning during embryogenesis [73].

In a subcutaneous model using severe-combined immunodeficient (SCID) mice, adenoviral-mediated forced expression of *SMAD4* in several PCCs that are devoid of *SMAD4* resulted in suppressed tumor growth in conjunction with decreased expression of vascular endothelial growth factor (VEGF) and gelatinases [74]. In subcutaneous nude mouse models of PDAC, reexpression of *SMAD4* in Hs766T PCCs confirmed that tumor growth was attenuated and angiogenesis was decreased due to attenuated VEGF expression [75], whereas in BxPC3 PCCs tumor growth was

attenuated as a consequence of decreased PCC proliferation without evidence for decreased angiogenesis [76]. Moreover, with time, BxPC3 cells were able to escape TGF-β's growth suppressive effects resulting in accelerated growth [76]. Inasmuch as all three studies used a subcutaneous model, their results must be interpreted with caution, since subcutaneous mouse models of PDAC are known to be very vascular and are not believed to represent PDAC in humans.

A vastly improved genetically engineered mouse model (GEMM) of PDAC was reported in 2003 that recapitulates many features of human PDAC. Thus, activation of oncogenic *Kras* transcription occurs through its endogenous promoter when mice carrying a *LoxP-Stop-LoxP* element (*LSL*) upstream of the silenced transcriptional start site of the $Kras^{G12D}$ allele are crossed with mice carrying a pancreas-specific promoter that drives Cre recombinase to excise the *LSL* site [77]. Generation of the KC GEMM, which expresses oncogenic *Kras* driven by Cre recombinase, yields animals that develops pancreatic intraepithelial neoplasia (PanIN) and acinar to ductal metaplasia (ADM) lesions by 2 months of age, and PDAC at variable penetrance by 8 to 12 months of age [77]. PanIN are seen in both human PDAC and GEMMs of PDAC and progress at variable rates from PanIN-1 to PanIN-2 and -3 [77, 78]. Progression to PDAC is accelerated when the KC GEMM is modified by conditional loss of tumor suppressor genes such as *Ink4a* or *Trp53* [78]. For example, *Pdx1-Cre*;*LSL-*$Kras^{G12D}$;$Ink4a^{lox/lox}$ mice harbor oncogenic $Kras^{G12D}$ and a homozygous deletion of the *Ink4a* locus, resulting in large, highly invasive ductal adenocarcinomas with frequent EMT changes by 7–11 weeks of age [78]. By contrast, *Pdx1-Cre*;*LSL-*$Kras^{G12D}$;$Smad4^{lox/lox}$ mice exhibit acceleration of tumor progression [79], but these tumors resemble intraductal papillary mucinous neoplasia (IPMNs). *Smad4* deficiency also accelerated PDAC development in *Pdx1-Cre*; *LSL-*$Kras^{G12D}$;$Ink4a^{lox/+}$ mice but the cancer cells appeared to be better differentiated than the corresponding GEMM with wild-type Smad4 [79]. Importantly, some PCC lines established from the *Pdx1-Cre*;*LSL-*$Kras^{G12D}$;$Ink4a^{lox/+}$ mice expressing wild-type *Smad4* exhibited enhanced proliferation in response to TGF-β [79], underscoring TGF-β's potential to act as a mitogen in some PCCs.

GEMMs have transformed our knowledge of PDAC pathobiology. For example, studies with GEMMs in which EGFR deletion in the pancreas abrogated cancer formation in oncogenic Kras-driven models [80] underscored the importance of EGFR family members in PDAC pathobiology in spite of the presence of *Kras* mutations in these models. Specifically in regards to the *Pdx1-Cre*;*LSL-*$Kras^{G12D}$; $Smad4^{lox/lox}$ mice, it was shown that loss of expression of the Anterior gradient 2 (*Agr2*) gene delays PanIN initiation and progression to PDAC [81], suggesting that loss of *Smad4* may convert TGF-β from a tumor suppressor that can decrease AGR2 expression to a tumor promoter that upregulates AGR2 expression [81].

Smad7 and MicroRNAs

In a feed-forward loop, TGF-β enhances miRNA-21 expression that, in turn, leads to more sustained TGF-β signaling by downregulating the levels of inhibitory Smad7

[82]. Moreover, miRNA-106b and miRNA-182b also target Smad7 [83, 84], suggesting that downregulation of Smad7 may be an important mechanism for enhancing TGF-β's tumor promoting actions. In support of this possibility, oncomirs, such as miRNA-372, and miRNA-302 attenuate TGF-β signaling by targeting TβRII [85], suggesting that suppression of TGF-β actions is a widespread mechanism in oncogenesis. However, it is not clear at what step during neoplastic transformation TGF-β converts from a tumor suppressor to a tumor promoter. Moreover, hypoxia, tumor necrosis factor alpha (TNF-α), interferon gamma (IFNγ), and mitogenic growth factors such as epidermal growth factor (EGF) act to upregulate Smad7 expression [86], and this upregulation occurs in the context of established tumors, when TGF-β functions as a tumor promoter.

The above observations raise the possibility that Smad7 actions in vivo may be context-dependent and may contribute to tumor progression. Indeed, in vivo studies indicate that Smad7 actions in tumors are more complex than in cultured cell lines. Thus, in human colon adenocarcinoma cells Smad7 attenuates TGF-β-induced G1 arrest and Akt phosphorylation while increasing TGF-β effects on c-Jun phosphorylation, and promoting anchorage-independent growth and tumorigenicity in nude mice [86]. Concomitantly, Smad7 inhibits p21 expression and apoptosis, as well as TGF-β-mediated suppression of Cyclin D1 and CDK4 [87]. In addition, Smad7 prevents TGF-β from maintaining pRb in an active, hypophosphorylated state [87].

In PCCs engineered to overexpress Smad7 there is an increase in thioredoxin levels, as well as enhanced anchorage-independent growth and tumor growth in vivo [88]. Moreover, laser capture microdissection followed by quantitative reverse-transcriptase PCR of RNA isolated from cancer cells in human PDAC samples revealed that thioredoxin and Smad7 are concomitantly overexpressed in PCCs in vivo, suggesting that thioredoxin is downstream of Smad7 in a pathway that promotes pancreatic cancer growth [88]. In support of this conclusion, Smad7 overexpression, by interfering with pRb functions in PCCs, derepresses E2F and enables TGF-β to promote tumor growth while blocking its growth inhibitory effects [54, 55]. These observations suggest that the in vivo consequences of the complex regulatory networks governing TGF-β-Smad4-Smad7 interactions are context-dependent and require further elucidation through the use of in vivo autochthonous models.

Paracrine Growth-Promoting Actions of TGF-β in PDAC

PDAC is associated with increased expression of all three TGF-β isoforms, and this overexpression in treatment-naïve patients who have undergone resection without receiving postoperative adjuvant therapy has been correlated with decreased patient survival [89]. It has been proposed that together with the loss of the tumor suppressor functions of TGF-β, its overexpression by PCCs in vivo drives PDAC progression by exerting paracrine actions in the tumor microenvironment (TME) that include alterations in the components of the extracellular matrix, enhanced stroma

formation, stimulation of aberrant angiogenesis, suppression of cancer-directed immune pathways, enhanced EMT, and a greater propensity for cancer cell to invade and metastasize.

An example of the complex paracrine actions by TGF-β is represented by its ability to induce stroma formation and to promote the expression of connective tissue growth factor (CTGF), a factor that also enhances stroma formation in PDAC. The importance of this TGF-β-CTGF axis in PDAC is underscored by the observations that blocking CTGF by an antibody (e.g., FG-3019) reduces tumor growth, metastasis, and angiogenesis in an orthotopic mouse model of PDAC [90] and in a genetically engineered mouse model (GEMM) of PDAC [91].

Direct Mitogenic Actions of TGF-β in Pancreatic Cancer Cells

As described earlier, TGF-β-mediated growth inhibition is dependent on the upregulation of CDK inhibitors such as $p21^{Cip1}$. The final common pathway downstream of these growth-inhibitory signals leads to the activation of pRb by maintaining it in a hypophosphorylated state. However, overexpression of Smad7 occurs in about 50% of PDACs, and this overexpression prevents TGF-β from inhibiting proliferation [88]. Moreover, the PCCs in PDAC tissues exhibit strong phospho-pRb, Ki67, and phospho-Smad3 immunoreactivity but low level of $p21^{Cip1}$ [55], suggesting that activation of TGF-β pathways is not associated with $p21^{Cip1}$ upregulation and fails to inhibit PCC proliferation in PDAC in vivo. Low levels of $p21^{Cip1}$ in conjunction with loss of pRb function are also associated with enhanced PCC proliferation in spite of the expression of markers generally associated with senescence [54]. In theory, the senescence response prevents the proliferation of dysfunctional cells that have the potential to undergo malignant transformation, and the cells exhibit β-galactosidase activity at pH 6.0 in contrast to being active at pH 4.0 as observed in lysosomes. Enhanced proliferative capacity occurring in this context has been termed senescence bypass.

The mechanisms whereby TGF-β can exert direct mitogenic effects on PCCs was elucidated in a study in which PCCs were isolated from a GEMM of PDAC that was generated by using mice that express *Rb* with a floxed STOP codon in exon 19 that were crossed with mice carrying *LSL-Kras*^{G12D} and *Pdx1-Cre* [54, 55]. This breeding strategy yielded compound mutant mice expressing *Kras*^{G12D} in the context of loss of pRb. PCCs established from this KRC GEMM express senescence markers but are hyperproliferative due to the loss of pRb and low $p21^{Cip1}$ levels, indicting that they have undergone senescence bypass [55]. Moreover, these changes occur in conjunction with a robust senescence-associated secretory phenotype with production of multiple cytokines and elevated TGF-β levels [54, 55].

Importantly, TGF-β enhances proliferation in PCCs derived from the KRC GEMM, and this effect is especially pronounced when the cells are grown in three-dimensional (3D) cultures [55].

Noncanonical TGF-β Actions in Pancreatic Cancer

TGF-β's direct mitogenic effects in PCCs that are devoid of pRb are mediated by the activation of noncanonical TGF-β pathways, such as extracellular signal regulating kinase (ERK), AKT, and Src [55]. Moreover, restoring wild-type pRb expression in these cells, but not mutated pRb, eliminates TGF-β's growth stimulatory actions [55], suggesting that the altered transcriptome that occurs as a consequence of the derepression of the E2F family of proteins preferentially redirects TGF-β signaling toward it's noncanonical pathways.

Noncanonical signaling can be activated by TGF-β through a variety of mechanisms (Fig. 2). For example, TGF-β can induce TβRII autophosphorylation on tyrosine residues 259, 336, and 424 [92], and can directly phosphorylate tyrosine residues on TβRI as well as serine and tyrosine residues on SchA [93]. Importantly, SchA phosphorylation results in the formation of ShcA/Grb2/Sos complex that can activate Ras and multiple downstream signaling cascades that promote tumorigenicity [93].

An altogether different mechanism is demonstrated by the actions of TGF-β-activated kinase 1 (TAK1) since it activates mitogen-activated kinase kinase

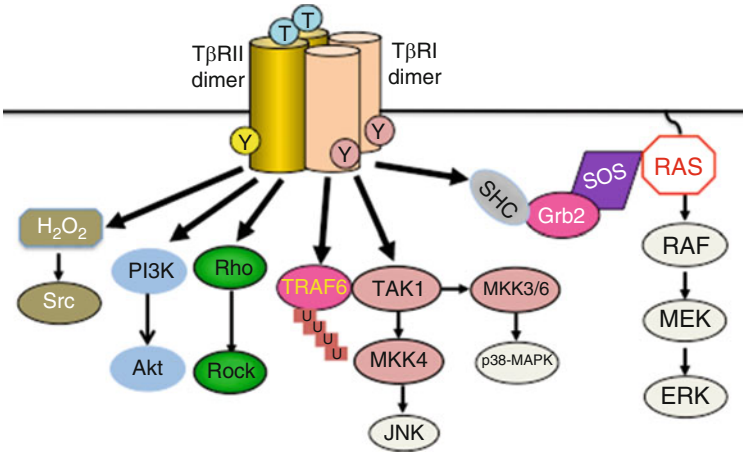

Fig. 2 Noncanonical TGF-β signaling. Both TβRII and TβRI can be phosphorylated on tyrosine residues, enabling the p85 regulatory subunit of PI3K to associate with either receptor, leading to the activation of PI3K and Akt. TGF-β also directly phosphorylates serine and tyrosine residues on SchA. SchA phosphorylation generates ShcA/Grb2/Sos complexes that activate Ras and multiple downstream signaling cascades. In addition, TGF-β-activated kinase 1 (*TAK1*) is activated as a result of its association with K63-linked poly-ubiquitilation (shown as a chain of small boxes labeled u for ubiquitin) TRAF6 (TNF receptor-associated factor 6). TAK1 then activates mitogen-activated kinase kinase 6 (*MKK6*) and MKK3, which then activate p38-MAPK. TAK1 also activates mitogen-activated kinase kinase 4 (*MKK4*) that activates Jun kinase (*JNK*). TGF-β also activates Rho GTPase, thereby activating its downstream effector Rock. Moreover, TGF-β activates NADPH oxidase, thereby generating H_2O_2 (hydrogen peroxide) leading to the activation of Src

6 (MKK6) and MKK3, which then activate p38-MAPK, thereby leading to enhanced phosphorylation of Smad2 [94]. TAK1 also activates mitogen-activated kinase kinase 4 (MKK4) that activates Jun kinase (JNK), and JNK is known to promote mitogenesis [94]. Moreover, the activation of TAK1 requires the presence of a modified form of TNF receptor-associated factor 6 (TRAF6) that must initially associate to active TβRI and undergo K63-linked poly-ubiquitilation chains on TRAF6 prior to being capable of recruiting of TAK1 [94]. Intriguingly, p38-MAPK immunoreactivity in PDAC has been correlated with better prognosis [95]. Taken together, these observations underscore the complex nature of the TAK1 noncanonical pathway.

There are several additional noncanonical pathways, which include activation of PI3K, Rho GTPase, and Rho-like GTPases such as Rac and Cdc42 [28]. Moreover, as a consequence of its ability to activate NADPH oxidases, TGF-β has been shown to increase hydrogen peroxide (H_2O_2) generation and thereby lead to the activation of Src [96], as depicted in Fig. 2.

Therapeutic Implications

Reagents for Targeting TGF-β

Multiple strategies have been proposed to suppress TGF-β deleterious effects in cancer. Some have worked in cell culture systems and subsequently in preclinical models. Moreover, some have moved into clinical trials, but a great deal of work remains to be done to find the right strategy that successfully treats a cancer and does not cause major side effects.

At the ligand level, approaches for targeting TGF-β have ranged from the use of anti-TGF-β neutralizing antibodies to impede TGF-β actions [97], antisense mRNAs or antisense oligonucleotides to inhibit TGF-β synthesis [98], a dominant-negative form of the TGF-β1 precursor to inhibit TGF-β isoform processing [99] soluble TβRII and TβRIII that act to sequester TGF-βs [100], and monoclonal antibodies that target all three TGF-β isoforms [101]. At the receptor level, a variety of screening strategies generated numerous small molecule inhibitors of the kinase activity of TβRI (206–214). At the postreceptor level, a variety of strategies have been shown to target components of downstream signaling pathways.

Preclinical Studies of Targeted TGF-β Therapy in PDAC

Expressing a soluble TβRII (sTβRII) construct in PCCs led to attenuated tumor growth in a subcutaneous nude mouse model as well as attenuated tumor growth, metastasis, and malignant ascites formation in an orthotopic mouse model of PDAC [101]. Expression of sTβRII also attenuated tumor angiogenesis and lowered the levels of mRNA moieties encoding plasminogen activator inhibitor 1 and urokinase plasminogen activator, both of which have been implicated in tumor growth and

metastasis [101]. Moreover, the small molecule inhibitor LY2109761, which targets both TβRI and TβRII, was shown to suppress pancreatic cancer metastasis in an orthotopic model [102]. Orthotopic mouse models have therefore helped to demonstrate the potential benefits of targeting TGF-β in PDAC.

By contrast to the findings in orthotopic models, in a GEMM of PDAC in which oncogenic *Kras* was combined with heterozygous loss of *Tp53*, TGF-β targeting using a monoclonal antibody that inhibits the actions of all three TGF-β isoforms (1D11), resulted in enhanced PDAC progression [103], casting doubt on the potential benefit of targeting TGF-β in PDAC. The same study also demonstrated that the integrin αvβ6 may contribute to TGF-β's tumor suppressor function and that targeting αvβ6 with a highly specific monoclonal antibody also accelerates PDAC progression in this GEMM [103]. However, with both strategies, accelerated tumor growth was dependent on the presence of wild-type *Smad4* [103], and 1D11 has been shown to inhibit pulmonary metastases in a murine mammary cancer model while also promoting antitumor immune mechanisms by enhancing the activity of CD8+ T cells [102]. Furthermore, it has been recently demonstrated that TGF-β can induce both EMT and apoptosis in PCCs that express wild-type Smad4, but promote tumor progression in PCCs devoid of Smad4 [104], underscoring the context-dependence of potential responses to TGF-β-targeted therapies.

TGF-β and Angiogenesis in PDAC

PDACs are generally hypovascular and desmoplatic. However, PDACs are also heterogeneous and may exhibit regions of readily detectable microvasculature. Thus, in addition to being hypoxic, PDACs can have blood flow and obtain necessary nutrients from the arterial blood supply while discharging waste into their venous drainage.

Using TCGA-derived RNA-seq data it was determined that ~35% of PDACs expressed a strong pro-angiogenesis gene signature, and the same PDACs expressed a transcriptome indicative of active TGF-β and pro-inflammatory signaling pathways that was similar to that observed in the KRC GEMM [105]. Expression of Smad4 correlated with the presence of a TGF-β gene signature in these samples [105]. An additional 47% of PDACs exhibited a moderate pro-angiogenic gene signature that was similar to that observed in the KRC GEMM [105]. Only ~18% of the PDACs expressed very few pro-angiogenic genes, and this signature was very similar to the gene signature seen in the highly desmoplastic and hypovascular KPC tumors. Taken together, these observations suggest that PDACs exhibit marked heterogeneity in relation to tumor angiogenesis, and TGF-β may exert multiple paracrine actions on the TME in PDAC (Fig. 3), by promoting aberrant angiogenesis and desmoplasia, suppressing cancer-directed immune mechanisms, and directly enhancing PCC survival and proliferation. Therefore, proposed therapeutic approaches need to consider these multifaceted actions of TGF-β when designing clinical trials.

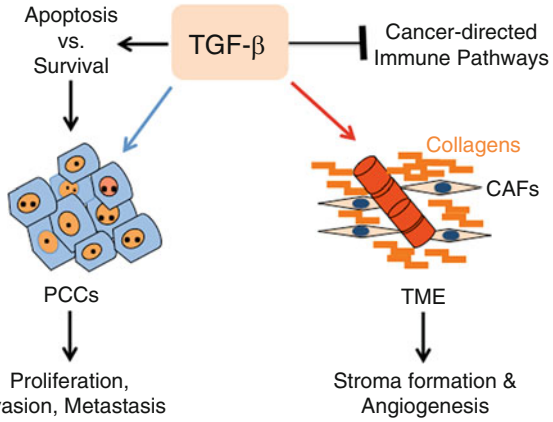

Fig. 3 Paracrine TGF-β actions in PDAC. PDAC overexpresses all three human TGF-β isoforms all of which are released into the tumor microenvironment where they promote the proliferation of cancer-associated fibroblasts (*CAFs*), induce deposition of collagen and fibronectin to help generate PDAC-associated desmoplasia, and stimulate tumor micro-angiogenesis. In addition, TGF-βs can promote the survival and proliferation of pancreatic cancer cells (*PCCs*), and enhance their invasiveness and metastatic potential, while suppressing cancer-directed immune mechanisms

Future Directions for Targeting TGF-β in PDAC

The explosive generation of new data thanks to novel technologies and "omics" ranging from genomics, to proteomics, metabolomics, lipidomics, epigenomics (as just some examples) combined with powerful informatics tools and supercomputing, high-throughput screening strategies, and novel drug packaging and delivery methodologies will allow for novel combinatorial therapies with fewer side effects, dramatic improvements in efforts at delivering effective precision medicine. In addition, novel strategies for early diagnosis and improvements in our understanding of why immune checkpoint inhibitors have not been as successful in PDAC as in some other solid tumors will allow for earlier and more successful immune- and vaccine-based interventions that will dramatically increase cure rates. In this regard, it is important to understand that TGF-β is both a tumor suppressor and a tumor promoter, depending on the stage of PDAC development. Moreover, recognizing that TGF-β plays a pivotal role in immune modulation, it will be important to continue to advance our knowledge regarding TGF-β-mediated cell-autonomous and paracrine actions, and to improve our understanding of the intersections of these pathways with other canonical and noncanonical signaling cascades, the immune system, and noncoding RNAs.

Conclusion

TGF-β exerts important regulatory actions in the normal pancreas where it functions as a tumor suppressor. However, TGF-β actions are context dependent. Thus, in the presence of major driver mutations that are common in PDAC, such *KRAS*, *TP53*,

and *SMAD4* mutations, TGF-β converts from a tumor suppressor to a tumor promoter, exerting deleterious paracrine actions on the tumor microenvironment and promoting immune evasion. In addition, TGF-β can exert direct mitogenic effects on pancreatic cancer cells by activation of noncanonical signaling pathways, and the capacity to exert these effects is enhanced as a result of the functional inactivation of pRb caused by excessive mitogenic signaling, *KRAS* mutations, loss of *CDKN2A*, and Smad7 overexpression. TGF-β also interacts with signaling pathways that are downstream of other members of the TGF-β superfamily and/or downstream of tyrosine kinase receptors. Consequently, strategies designed to suppress TGF-β signaling in PDAC need to consider the mutational landscape and specific signaling nodes that are active within a given tumor in order to allow for safe and effective combinatorial therapies.

Key Research Points

TGF-β signaling is complex, context-dependent, and regulated by positive and negative signaling inputs.

TGF-β signaling is mediated via canonical and noncanonical pathways.

In the normal pancreas, TGF-β acts as a tumor suppressor and functions to maintain acinar cell homeostasis.

In PDAC, TGF-β exerts paracrine effects on the tumor microenvironment to enhance PDAC growth and metastasis, but it can also exert direct mitogenic effects on pancreatic cancer cells.

Future Scientific Directions

There is a need for improved understanding of TGF-β signaling and its cross-talk pathways.

There is a need for improved understanding of TGF-β-mediated suppression of cancer-directed immune mechanisms and how this might impact attempts at immunotherapy.

It is important to gain a better understanding of the direct mitogenic effects of TGF-β on pancreatic cancer cells in order to improve therapeutic strategies.

Clinical Implications

Effective therapies aimed at targeting TGF-β pathways require precision medicine tools in order to avoid loss of TGF-β's tumor suppressor functions.

Combinatorial therapies that target TGF-β pathways and other deleterious pathways may restore cancer-directed immune mechanisms and improve immune checkpoint inhibition therapies.

It is crucial to understand tumor heterogeneity issues in relation to TGF-β actions in order to effectively target deleterious TGF-β pathways without enhancing progression of precancerous lesions.

Cross-References

- ▶ Approaching Pancreatic Cancer Phenotypes via Metabolomics
- ▶ Differential Therapy Based on Tumor Heterogeneity in Pancreatic Cancer
- ▶ Emerging Therapeutic Targets in Pancreatic Adenocarcinoma
- ▶ Epigenetic Pharmacology
- ▶ Metabolism in Pancreatic Cancer
- ▶ Precision Medicine Based on Next-Generation Sequencing and Master Controllers

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Notch Signaling in Pancreatic Morphogenesis and Pancreatic Cancer Pathogenesis

Gwen Lomberk and Raul Urrutia

Contents

Abstract

Notch signaling is the focus of investigation in a large number of laboratories around the world due to its pleiotropic effect in regulating normal development and alterations in cancer. During the last few decades, the scientific community studying this pathway has made significant contributions to our understanding of the cellular role of Notch signaling in regulating proliferation, differentiation,

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apoptosis, migration, branching morphogenesis, and angiogenesis. Similar to observations with other signaling cascades, such as TGBβ, besides its role in morphogenesis, Notch signaling becomes dysregulated in adult tissue and contributes to the development and maintenance of the cancer phenotype. Elegant studies in this field of research have led to not only the better understanding of the molecules within the pathway but, as a consequence, rational design of drugs that can inhibit Notch signaling with promising results. The study of Notch signaling in the pancreas has dawned on solid ground and has progressed to a better understanding of the pathway at the mechanistic level along with the development of some promising pharmacological antagonists. Thus, investigations in this field are predicted to continue to advance the field of pancreatic cancer research in a significant manner for decades to come.

Keywords
Notch · Morphogenesis · Development · Signaling · Pancreatic cancer · γ-Secretase

Introduction

Since the discovery of mutant Notch phenotypes in the fly wing over 100 years ago [1], Notch signaling has continually elicited significant attention from the basic science community because of its ability to regulate normal morphogenesis in a conserved manner from flies to human. This remarkable conservation throughout the animal kingdom suggests that evolution has exercised a strong pressure for maintaining this morphogenetic cascade for millions of years, thus underscoring its importance for life. Almost seven decades after the first observation of the notched wing phenotype, the Artavanis-Tsakonas and Young labs independently cloned the Notch receptor, finally attributing this phenotype to gene haploinsufficiency [2, 3]. From this work, studies on the Notch pathway have propagated a revolution in a large number of fields, including developmental and stem cell biology, neuroscience, as well as cancer biology [4]. Developmentally, Notch signaling became first known as a robust mediator of lateral inhibition, a key patterning process that organizes the regular spacing of different cell types within tissues, including branching morphogenesis of a similar type as that observed in the pancreas [5–8]. In fact, several molecules from the Notch signaling pathway are potent regulators of normal pancreas organogenesis and/or neoplastic transformation in this organ. Initially, the interest of Notch signaling as a modulator of disease states developed from studies of its role in hereditary diseases that result from abnormal morphogenesis, such as Alagille syndrome, spondylocostal dysostosis, and several cancers, all of which display aberrant ligand expression [9–11]. However, in the adult pancreas, Notch has also been shown to recapitulate some of its developmental functions, thus aiding in both regeneration [12] and the acquisition of the neoplastic phenotype [6, 8, 13]. As a result, the current concept is that Notch signaling is associated not only with pancreatic morphogenesis but also with the development and/or maintenance of the pancreatic cancer cell phenotype.

The attractiveness of studying this pathway for pancreatic cancer investigators is due to an increased need to better understand the pathobiological role of this type of signaling in pancreatic cancer along with the relative ease that exists for pharmacologically targeting this pathway, which has led all the way to clinical trials. This increased understanding on how Notch signaling regulates an aggressive cancer phenotype in this organ, at the fine cellular and molecular level, is very promising to derive potential "biomolecular-based therapeutic modalities" that can be combined with the currently existing therapies to fight this disease. Thus, in this chapter, the current knowledge in the field of Notch signaling research is updated, and a theoretical framework that covers the molecular to the pathobiological role of this biochemical cascade in pancreatic cells is discussed.

Notch Receptor

Receptors of the Notch family are cell-surface type I transmembrane proteins, consisting of four members (Notch −1, −2, −3, and −4). Upon ligand binding, Notch receptors undergo successive proteolytic cleavages that lead to the release of the Notch intra-cellular domain (NICD) (Fig. 1) [6, 14]. This cleaved Notch intracellular domain is the active form of the receptor. In fact, several studies have shown that this pathway can be activated, in a ligand-independent manner, by simply overexpressing the NICD.

In order to better understand the mechanism of Notch signaling, it is important to remember the domain composition of this receptor since its interaction with other proteins, including ligands, depends upon this structural composition and domain organization [14]. Most notably, the extracellular domain of Notch is composed of 36 EGF repeats in vertebrates, though their number varies according to the organism being considered (Fig. 2). Another important motif includes three Lin12/Notch repeats. Careful biochemical analysis has demonstrated that the repeats 11 and 12 EGF function as binding sites for Delta and Serrate [15]. The Notch intracellular domain includes six ankyrin repeats and two classically basic residue-charged nuclear localization signals. The positions of the S1–S4 cleavage sites are crucial, since cleavage at these sites, which is achieved by the γ-secretase enzyme, releases the intracellular domain [16]. In turn, the intracellular domain subsequently migrates to the nucleus to function as a transcriptional regulator. Thus, this system appears to have evolved to mediate the characteristic long-term transcriptional response that is necessary to trigger a hierarchical cascade of gene expression, responsible of regulating cell differentiation, tissue remodeling, and morphogenesis.

Notch Ligand

Initially, most of the mechanistic information gained about the Notch signaling pathway was gathered from experiments in *Drosophila melanogaster* [17]. The two ligands found as results of these experiments, Delta and Serrate, and

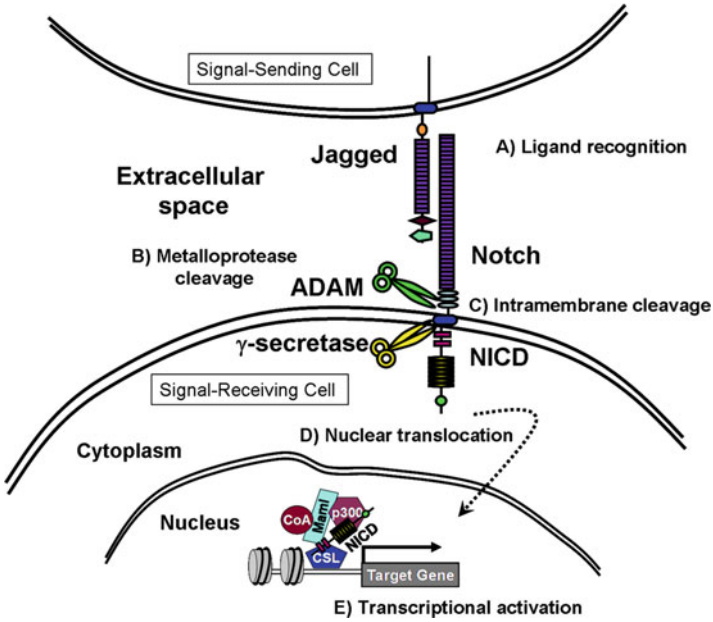

Fig. 1 **The Notch signaling pathway**. The figure illustrates the key events in the Notch signaling pathway. Ligands of the delta and jagged families expressed on an adjacent signal-sending cell initiate the signal through Notch receptor recognition on the signal-receiving cell (**a**). This interaction between receptor and ligand leads to a cascade of proteolytic cleavages of the Notch receptor, beginning with metalloprotease cleavage just outside the membrane (**b**). This proteolytic step facilitates the subsequent intramembrane cleavage of Notch by the γ-secretase complex (**c**) to release the Notch intracellular domain (*NICD*) from the membrane. The NICD then translocates to the nucleus (**d**) and enters into a transcriptional activation complex with the transcription factor CSL along with coactivators, including Mastermind-like proteins (Maml) and CBP/p300, thereby activating transcription of target genes (**e**)

Lag2, another molecule with similar domains, are known today as the canonical DSL (Delta, Serrate, Lag2) ligands, which are believed to be responsible for most Notch functions [15]. Noteworthy, however, noncanonical ligands have also been shown to activate Notch, though little is known about these pathways [18].

Similar to the Notch receptor, the canonical ligands are also type 1 cell-surface proteins containing tandem epidermal growth factor (EGF) repeats in their extracellular domains (Fig. 3). The DSL domain, the N-terminal (NT) domain, as well as the first two EGF repeats are required for binding of these ligands to Notch [19, 20]. The mammalian canonical ligands are identified by their homology to the two *Drosophila* ligands, Delta and Serrate, and are designated as either Delta-like (Dll1, Dll3, and Dll4) or Serrate-like (Jagged1 and Jagged2) [15]. The intracellular domain of DSL ligands contains a C-terminal PDZ motif [21], which is required for signaling and interactions with the cytoskeleton. The currently accepted model for Notch signaling activation is initiated at the cell membrane by the DSL ligand expressed in one cell (signal-sending cell) and a Notch receptor

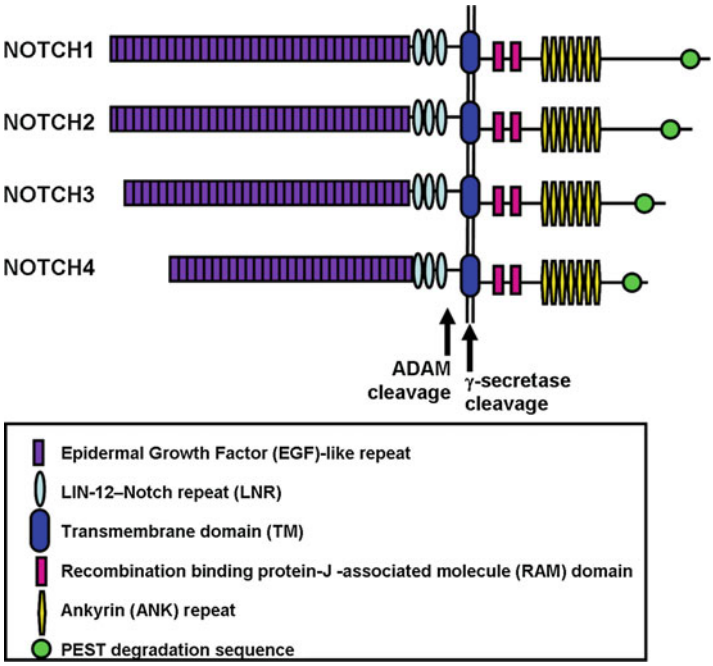

Fig. 2 The human Notch receptors. Schematic diagram of the structural domain features of the human Notch receptors 1–4. The *arrows* mark the approximate locations of the cleavage sites for the ADAM metalloprotease and γ-secretase for release of the NICD. The *double line* represents the cellular membrane. The legend box identifies the graphic representation of each structural feature

(Notch1–4) expressed on another cell in close proximity (signal receiving cell). Consequently, since cell-to-cell contact is necessary to activate this pathway, a Notch-bearing cell would be regulated by its neighboring cells expressing the Delta and Serrate ligands to achieve lateral inhibition. Lateral inhibition, as it has been classically described for early neuroblast differentiation, is a process in which Notch mediates reciprocal inhibitory signaling between neuroblasts that otherwise have a similar potential for cell phenotype determination [22]. In order to present Notch to ligand, these molecules form heterodimer produced as a result of processing by a furin-like protease during transit to the plasma membrane [23]. Ligand binding initiates additional cleavages of Notch, first by a disintegrin and metalloproteases (ADAM) within the juxtamembrane region, followed by γ-secretase within the transmembrane domain, thereby resulting in the release of the Notch intracellular domain (Fig. 1) [6, 24]. γ-Secretase is made of four subunits, namely presenilin (PS), APH-1, nicastrin, and PEN-2. PS is the catalytic peptide, which provides the aspartyl protease activity to the entire complex [25]. Therefore, pharmacologically inhibiting this process, as is commonly done using γ-secretase via pharmacological inhibitors [26], can disrupt Notch signaling, raising the possibilities that tools of this type can be used to manipulate the

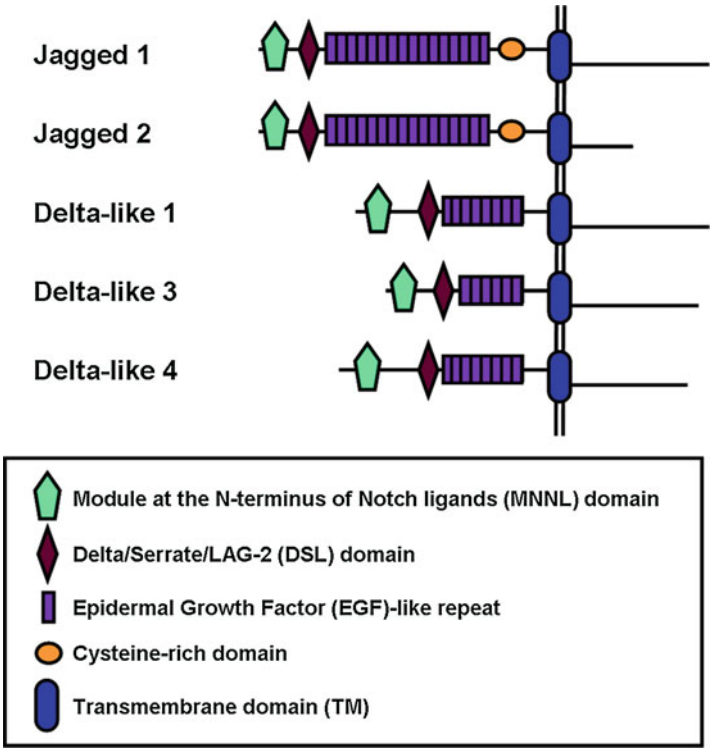

Fig. 3 The human DSL ligands. Schematic diagram of the structural domain features of the human DSL ligands for Notch with the *double line* representing the cellular membrane. The legend box identifies the graphic representation of each structural feature

pathway for therapeutic purposes, though its disruption is not necessarily cell specific and may have unwanted consequences.

Intracellular Signaling Molecules for the Notch Pathway: The first intracellular signaling peptide that must be considered the beginning of the Notch signaling pathway is the NICD, which is produced by proteolytic cleavages of the receptors. This peptide translocates to the nucleus and associates with the CSL (CBF1/Su(H)/Lag-1) family of transcription factor complexes (Fig. 1), resulting in subsequent activation of Notch target genes, such as *Myc*, *p21*, and HES, and Hey family members, via the mastermind-like transcriptional coactivators [27, 28]. *Hes* and *Hey* genes are the mammalian counterparts of the Hairy and Enhancer-of-split type of genes in *Drosophila,* and they represent the primary targets of the Delta-Notch signaling pathway [24, 29]. In this review, the primary focus is to describe the role of Hes proteins as Notch signaling molecules because of their role in pancreatic morphogenesis [30, 31]. In mammals, there are seven members in the Hes family (Hes1–7), although Hes4 is absent in the mouse genome.

Crosstalk with Other Signaling Cascades

Continuous tissue remodeling during embryogenesis requires coordinated regulation among many signaling pathways to maintain the balance between proliferation and differentiation, stem cells and immature progenitor cells. Canonical Notch signaling has long been regarded as a signaling cascade that is sufficient for morphogenesis. However, recent studies have shown that Notch signaling can establish a crosstalk with other cascades in order to achieve its pleiotrophic effects [32–35]. Some of these pathways include Hedgehog, TGFβ, BMP, VEGF, and Wnt signaling. Following, the crosstalk between Notch with TGFβ and VEGF signaling is described for two very important reasons: namely, these pathways are the best known Notch interactors and they both play an important role in cancer-related functions, such as angiogenesis.

Interestingly, most of the data regarding the role of Notch in angiogenesis has been derived from experiments in animal models. For instance, mice in which *Notch1* has been disrupted in the whole animal, by homologous recombination, are lethal at E10.5 because the primary vascular plexi in the yolk sac and brain undergo aberrant remodeling [36]. In addition, this phenotype also includes alterations in large vessels. Supporting the validity of this data, experiments performed using a vascular-specific knockout of *Notch1* displays remarkably similar defects [36]. Alterations in vascular biology have also been observed in genetically engineered animals expressing genes encoding proteins from the Notch signaling pathway [37]. For instance, knockout of a single allele of *dll4* leads to lethality at E9.5 also due to a failure in remodeling the primary vascular plexus. This phenotype is also recapitulated in jagged1-deficient mice and RBP-J–deficient mice. Additional experiments using a gain-of-function paradigm based on expressing the NICD have shown alterations in angiogenesis [38]. Thus, the role of Notch signaling in endothelial cell biology and angiogenesis is well established.

Notch-TGFβ Interactions

As mentioned above, under defined circumstances, Notch signaling has been found to interact with TGFβ signaling. Members of the TGFβ family of cytokines form distinct signaling subfamilies, including TGFβ, BMPs, Activin, and Inhibin, among others. Signaling via these cytokines begins at the cell surface by activating distinct serine/threonine kinases, which in turn transduce the intracellular signal to the nucleus through either Smad-dependent or independent mechanisms [39]. In endothelial cells, TGFβ induces cell migration while arresting proliferation [40]. In addition, many members of the TGFβ family of cytokines not only have the ability to display similar effects on endothelial cells but also stimulate pericytes, which are critical for vessel formation [41]. Therefore, the role of these cytokines in angiogenesis is recognized.

In the past several years, emerging evidence supports a role for an interaction among these pathways in angiogenesis. For instance, TGFβ induces endothelial cell migration in a manner dependent upon a pathway that involves Jagged-1-Notch-Hey-1-Smad3 [42]. TGFβ-mediated arrest of cell proliferation has been found to require Notch signaling [33]. Many TGFβ-inducible genes require an additional stimulation with Notch to achieve full expression. Knockout of the Notch ligand, Jagged-1, leads to a reduction of TGFβ-mediated induction of p21 and rescues the cell cycle arrest that is characteristic of this pathway [43]. Lastly, signaling via the intracellular domain of certain Notch receptors has been found to interact with Smads from the BMP pathway and appear to participate in signaling by this cytokine [42, 44].

Notch-VEGF Interactions

Basic evidence for an interaction between angiogenic factors and Notch signaling has been gathered by the observation that VEGF induces both Notch receptor and ligand [45]. For instance, notch1 and dll4 are upregulated by VEGF, via both VEGFR1 and VEGFR2, in human arterial cells [46]. This upregulation in Notch requires signaling through phosphatidylinositol 3-kinase/Akt, but not MAPK/ERK or src kinases. Interestingly, similar results have been found in the mouse retina, where VEGF induction of dll4 was demonstrated [47]. Administration of VEGF in mouse retinas increases expression of dll4, whereas injection of the VEGF antagonist, VEGF-Trap, downregulates the expression of this molecule. Noteworthy, however, Dll4 in this situation forms part of a negative feedback loop where Notch signaling upregulates HESR1 (HEY1), which then functionally interacts with SP1 sites to silence *VEGFR2* gene expression. At the cellular level, the *dll4*+/− phenotype increases filopodia and branching angiogenesis, which can be antagonized, at least partially, by reducing VEGF levels with sFlt1 (soluble VEGFR1 extracellular domain) or by blocking VEGFR2 using specific antibodies. These studies on dll4 in developing retina indicate that, in the presence of Notch signaling, cells may migrate toward a VEGF gradient in order to facilitate the initial steps of angiogenesis. Remarkably, subsequent downregulation of these signals correlate with subsequent steps in angiogenesis, such as anastomoses, tube formation, and vessel maturation. Lastly, the VEGF-Notch signaling interaction has been validated using zebrafish, *Danio rerio*, as an in vivo animal model [45]. In zebrafish, this pathway appears to underlie arteriovenous specification. In VEGF morphants, the dorsal aorta loses arterial markers, such as ephrin B2, and ectopically expresses the vein marker, Flt4. The aberrant arterial phenotype is rescued by activated Notch in VEGF morphants, but not conversely, by VEGF in Notch mutants. These experiments are extremely informative because, together, they locate Notch downstream of VEGF in zebrafish arterial specification.

The Notch-Hes Pathway in Pancreatic Morphogenesis

Through the analyses of many experiments performed in various organisms ranging from *Drosophila melanogaster* to human, Notch has been found to play several functions that are important for development, normal physiology, and diseases. These functions include but are not limited to cell proliferation, cell differentiation, apoptosis, cell migration, angiogenesis, and branching morphogenesis. Having this concept in mind, in fact, allows us to understand how Notch is of significant importance for both pancreatic development and carcinogenesis [6].

During development, the pancreas originates from the endodermal foregut epithelium as two primordial parts of the organ, namely the dorsal and ventral pancreatic buds, which fuse to form the entire gland. In both pancreatic buds, the epithelium gives rise to exocrine and endocrine cells: exocrine progenitors become acinar cells, which secrete digestive enzymes, whereas endocrine cells emigrate from the epithelium to form islets. The liver and biliary systems also originate from the endodermal epithelium of the foregut. Together, this data indicate that both systems share a pattern of branch morphogenesis, which is not only needed under physiological conditions, but during cancer development the biliary and pancreatic ducts give rise to similar type of cancers, both with extremely aggressive behavior. Therefore, it can be predicted that these malignancies may, at least in part, overlap in the molecular mechanisms that give rise to and maintain their cancer phenotype.

At the molecular level, in the developing pancreas, the Ptf1a transcription factor promotes exocrine cell differentiation [48], whereas the bHLH gene, *Ngn3*, mediates the differentiation of all types of endocrine cells [49], including α (glucagon-producing), β (insulin-producing), δ (somatostatin-producing), and PP (pancreatic polypeptide-producing) cells. The role of the Notch pathway in this phenomenon can be better understood via its relationship with Ngn3. The inactivation of the murine *Hes1* by homologous recombination triggers an upregulation of *Ngn3*, creating a bias toward endocrine cell differentiation and severe hypoplasia of the gland [50]. Further supporting a critical role of Notch in pancreatic development is the fact that similar phenotypes are observed after either knocking out the delta-like 1 (*Dll1*) ligand or the transcription factor that is an effector of Notch, namely RBP-J (recombination signal sequence-binding protein) [8, 51] showing accelerated differentiation of pancreatic endocrine cells, as well as by the overexpression of either *Ngn3* or the intracellular form of Notch3 (repressor of Notch signaling) [52]. Together, this data strongly suggests that the Dll1-Notch-RBP-J-Hes1 pathway inhibits premature endocrine differentiation.

Hes1 also antagonizes the function of Ptf1a, the master regulator of exocrine cell differentiation, by directly targeting the *Ptf1a* promoter and silencing its expression [5]. Moreover, expression of the intracellular domain of Notch inhibits acinar cell differentiation by antagonizing the function of Ptf1a [7, 30]. Thus, in summary, Notch-Hes1 signaling promotes the maintenance of pancreatic progenitor cells by antagonizing Ptf1a and Ngn3. However, in *Hes1*-null mice, Ptf1a and Ngn3 are

ectopically expressed in the common bile duct, leading to the formation of an ectopic pancreas [50]. Thus, this observation emphasizes that the biliary tree has similarity with the pancreatic buds at the molecular level, at least enough as to adopt a pancreas phenotype when key pancreas-specific regulators are expressed in these cells. This is not a trivial finding since this type of transdifferentiation is not a common event in every tissue type. Thus, both the biliary and the pancreas epithelium appear to go through a phase of "capacitation," in which the expression of key Notch-induced transcription factors is able to push their phenotype either way. The potential contribution of this concept to better understanding normal bile and pancreatic duct morphogenesis and their cancers is potentially very insightful, though it remains an underrepresented area of research.

Elegant studies in zebrafish have also been very useful for learning the role of Notch in pancreatic morphogenesis. For instance, activated Notch and Notch target genes impair zebrafish acinar cell differentiation [30]. In fact, strong evidence supporting a role for Notch in regulating exocrine pancreatic differentiation has been derived from this work on zebrafish embryos, in which Notch signaling is disrupted (homozygous *mindbomb* mutations) [30]. Mutant embryos appear to have accelerated exocrine pancreatic differentiation as compared with wild-type controls. Similar alterations were also observed after expressing a dominant negative *Suppressor of Hairless* [*Su(H)*]. Mechanistic studies, using transient transfection assays in COS7 cells involving a Ptf1-responsive reporter gene, demonstrated that Notch and Notch/Su(H) target genes directly inhibit Ptf1 activity. Thus, since Ptf1 is a critical regulator of acinar cell differentiation and zymogen gene expression, this work in zebrafish has not only defined a role for Notch in acinar cell differentiation but also provides at least one mechanism by which this pathway functions.

Notch and Pancreatic Cancer

While the Notch signaling pathway is required for the expansion of pancreatic progenitor cells, Notch signaling is mostly suppressed in the adult pancreas [52]. At this stage, active Notch signaling is confined to centroacinar cells, which is substantiated by Hes1 staining of human and mouse pancreas [53, 54], as well as a Notch-responsive reporter strain [55]. This same reporter strain allowed the detection of active Notch signaling in preneoplastic lesions, known as pancreatic intraepithelial neoplasia (PanIN), and tumor cells of pancreatic ductal adenocarcinoma (PDAC)-bearing mice. As the case with many embryonic pathways, functions that Notch performs during embryonic development are recapitulated, to some extent, during cancer. Consequently, after organogenesis, it is critical that this signaling pathway undergoes tight regulation in order to prevent aberrant signaling, which has the potential to lead to neoplastic transformation, as described in other organs. Initially discovered to play a role in T cell lymphoblastic leukemia due to the identification of a recurrent chromosomal translocation [56], Notch signaling has been demonstrated to be involved in the development of many hematopoietic and solid malignancies, including pancreatic cancer [57].

Early and continuing interest in the role of the Notch pathway in PDAC come from studies indicating that expression levels of members of the Notch signaling pathway, including receptors, ligands, and downstream targets, were increased in human pancreatic cancer compared to normal human pancreas by microarray and qPCR or normal pancreatic ductal epithelium by IHC, suggesting that the Notch pathway is active in PDAC [54, 58]. In a large, integrated genomic analysis study, performed in 2016, of 456 PDAC samples to define molecular subtypes of pancreatic cancer, 32 recurrently mutated genes from 10 pathways were identified; one of which was the Notch pathway [59]. Central to the most commonly mutated oncogene in PDAC, *KRAS*, Notch signaling is required for Ras-induced transformation of fibroblasts [60], as well as the related Hras-driven tumorigenesis in a mouse mammary tumor model [61]. Interestingly, through the use of genetically engineered mice (GEM), analysis of precursor PanIN lesions from the *Pdx1-Cre; LSL-KRAS*^{G12D} mouse model of PDAC initiation recapitulated Notch pathway activation, as evidenced by strong nuclear expression, accompanied by faint cytoplasmic expression of Hes1. Notably, nuclear expression was not observed in the normal ducts or islet cells of these animals, nor in these compartments in control animals [62]. Similar results were obtained in a zebrafish model of PDAC with eGFP-*KRAS*^{G12D} expression specifically driven to the pancreas, in which live imaging analysis of the exocrine pancreatic tissue revealed not only *KRAS*-positive cells but progressive activation of TGFβ and Notch pathways [63]. Furthermore, inhibition of Notch signaling in the *Pdx1-Cre*; *LSL-KRAS*^{G12D}; *p53*$^{lox/+}$ mouse model, which advances to PDAC with distant metastases, was shown to attenuate the progression of PanIN to PDAC, mainly through a reduction in the proliferation rate of premalignant cells [55].

Notch is also a mediator of cell transdifferentiation, similarly known as metaplasia [13, 50]. Its role in this process is essential for the field, since frank PDAC is thought to progress in a multistep fashion from ductular-like preneoplastic PanIN lesions with metaplastic components by the accumulation of distinct mutations in oncogenes and tumor suppressor genes. Mice overexpressing TGFα, as driven by the *Elastase I* promoter/enhancer in acinar cells, undergo a massive metaplasia where the pancreas is often replaced by ductular-like structures, known as acinar-to-ductal metaplasia (ADM), which have lost most of the acinar phenotype and are surrounded by a robust desmoplastic reaction [54]. These lesions undergo neoplastic transformation, a process that can be significantly accelerated by crossing the mice with *p53* null animals [64]. In these GEM models, the expression levels of Notch receptors, ligands, and target genes were higher in metaplastic ducts than in adjacent normal appearing tissue *in vivo* and in organ explants exposed to TGFα [54]. Thus, together, the evidence gathered from mice and human studies propose that postnatal expression of Notch signaling molecules occurs in the metaplastic pancreatic epithelium, which is a phenomenon that correlates with cancer development.

Nevertheless, additional studies utilizing GEM models have revealed both oncogenic and tumor suppressive roles for Notch signaling in PDAC development. For instance, in studies utilizing a Cre-dependent *Notch1* gain-of-function transgene, *Rosa26*$^{Notch1IC\text{-}IRES\text{-}GFP}$ with the *Kras*^{G12D} and a tamoxifen-inducible *Pdx1-Cre*^{ERT},

dual activation of Notch1 and mutant Kras significantly increased PanIN formation in these animals in comparison to mice with only mutant Kras activation [13]. In addition, activation of *Kras*^{G12D} in fully differentiated adult acinar cells with the *Elastase1-Cre*^{ERT2} model formed PanIN lesions, which was also enhanced in the presence of *Notch1* activation. However, using the *Pdx1-Cre*-driven mouse model in which oncogenic *Kras* is activated simultaneously with deletion of *Notch1* in the pancreas, the loss of *Notch1* resulted in increased tumor incidence and progression, implying that *Notch1* can function as a tumor suppressor gene in PDAC [65]. Studies focused on both Notch 1 and 2 in a similar model of *Kras*^{G12D}-driven pancreatic carcinogenesis (*Ptf1a*$^{+/Cre(ex1)}$) indicated that mice with loss of *Notch2*, but not *Notch1*, survived significantly longer, only very rarely developed PDAC with ductal differentiation, and presented with a switch of phenotype toward anaplastic pancreatic cancer with epithelial-mesenchymal transition [66]. This key role of Notch2 in PanIN progression and malignant transformation was associated with its regulation of Myc signaling. In wild type and *Kras*^{G12D} animals, *Notch1* and *Notch2* were prominently expressed in whole-tissue mRNA, whereas *Notch3* and *Notch4* had comparatively low expression [66]. Furthermore, increased expression of *Notch2* and the Notch target gene, *Hes1*, but not *Notch1* was observed in *Kras*^{G12D} animals at an age when only a few PanIN1 lesions are notable, which corroborated prior reports [54]. In experiments aimed at assessing Notch1 as a regulator of *Kras*^{G12D}-driven ADM, utilizing both the *Pdx1-Cre* and *Elastase1-Cre*^{ERT2} models, oncogenic Kras was sufficient to drive ADM both in vitro and in vivo, but loss of *Notch1* has almost no impact on this process [67]. Similar to the studies from Hanlon, et al. [65], the number, but not the severity, of *Kras*^{G12D}-induced PanIN lesions was higher in mice with *Notch1* deletion [67]. Thus, in these contexts, *Notch1* deletion appears to make acinar cells more susceptible to formation of PanINs. Interestingly, knockout of *Hes1* in the *Ptf1a*$^{+/Cre(ex1)}$*; LSL-KRAS*^{G12D} model resembles features of the *Notch2* knockout animal reported by Mazur, et al. [66], with enhanced ADM formation and tumor development, but inhibition of high-grade PanIN formation [68]. When the *Ptf1a*$^{+/Cre(ex1)}$*; LSL-KRAS*^{G12D} model was crossed to mice carrying a dominant negative form of the Mastermind-like 1 gene, *MAML*, an essential coactivator of canonical Notch signaling-mediated transcription independent of which Notch receptor is activated, epithelial Notch signaling was inhibited and delayed PanIN initiation, but this effect was lost with age [69]. Collectively, these studies emphasize the importance of Notch signaling levels in the exocrine pancreas to maintain homeostasis, and the various observed cell-type and context-dependent effects of this pathway upon genetic manipulation should be considered when evaluating Notch inhibition for PDAC therapies.

The modes of action of Notch signaling pose an obvious mechanistic relevance of this pathway for tumor–stromal interactions. As mentioned in a previous section, the mechanism whereby Notch is expressed in a cell population in a manner that regulates cell fate is known as lateral inhibition. Therefore, the expression of Notch and its ligands can influence expression in neighboring cells. Notch also influences lineage decisions in more differentiated states in a manner that two daughter cells undergo asymmetric inheritance of ligands. Furthermore, Notch

ligands and receptors are expressed on different cell types, such that Notch can only be activated in the receptor-bearing cell in a mechanism of inductive signaling. This type of signaling, which can serve as a boundary between two groups of cells, has been modeled in tumor and stromal cell interactions. The unrelenting chemoresistance seen in PDAC is simultaneously influenced by tumor parenchymal and stromal factors. In studies evaluating critical pathways in tumor-stromal interactions, Fujita et al. found that direct coculture of pancreatic cancer cells and pancreatic stellate cells (PSCs) dramatically increased the mRNA levels of *Hes1* in both cell types, suggesting that direct cell contacts activated Notch signaling [70]. Another report from Cao and colleagues determined that a Notch pathway inhibitor or *Hes1* siRNA reversed the chemoresistance induced by PSCs and that high Hes1 levels are associated with poor prognosis in patients with PDAC [71]. Overactivation of the Notch pathway via the ligand Delta-like 4 (DLL4) enhanced the expression of genes associated with the epithelial–mesenchymal transition (EMT) and cancer stem cell (CSC) phenotypes, as well as induced multi-chemoresistance *in vitro* and inefficient chemo-drug delivery *in vivo* [72]. Therefore, therapy targeting the Notch signaling pathway has the potential to reverse chemoresistance and improve survival in patients with pancreatic cancer.

The Notch pathway has been found to play a role in additional mechanisms related to chemoresistance, such as elevated cancer stem cells (CSCs). Representing a small subpopulation of pancreatic cancer cells, CSCs are associated with an aggressive tumor behavior. The Notch pathway has been found to be further upregulated in CSCs compared to bulk pancreatic cancer cells [73]. Functionally, inhibition of the Notch pathway by a γ-secretase inhibitor or *Hes1* shRNA results in a reduction of pancreatic CSC self-renewal and tumorigenicity [73]. In contrast, use of an exogenous Notch peptide ligand to activate the pathway enhanced the percentage of CSCs and tumorsphere formation. Treatment of orthotopic PDAC tumors with a γ-secretase inhibitor not only inhibited tumor growth but also reduced the number of CSCs in these tumors [73]. Further studies have suggested that contributing factors to the failure of treatment in PDAC may be an increase in number of CSCs, as well as activation of the Notch pathway [74]. The evidence supporting a role of the Notch pathway in CSCs provides an additional rationale for targeting this pathway as a potential therapy for PDAC.

The key role of Notch signaling during pancreatic carcinogenesis has elicited interest in finding molecules capable of downregulating this pathway to normal levels, as potentially useful in the therapy of pancreatic cancer. Several suitable molecules have long existed from studies on the biology of Notch signaling, such as γ-secretase inhibitors, though new agents are under investigation, as discussed further in the following section. For instance, reports using cultured pancreatic cancer cells have shown that in BxPC-3, HPAC, and PANC-1 pancreatic cancer cells, Notch-1 downregulation causes the upregulation of NF-κB, a potential downstream target of this pathway and induces apoptosis [75]. In this work, the authors found that naturally occurring molecules (substances of great interest to the field of chemoprevention), such as genistein, are efficient in downregulating Notch signaling, thus adding to the arsenal of compounds that may serve as the foundation for

developing several generations of new drugs, which can be tested for either the chemoprevention of pancreatic cancer at the PanIN stage or even later when a frank tumor develops.

Targeting the Notch Pathway

Different types of small drugs, such as ADAM inhibitors, Notch antisense, anti-Notch monoclonal antibodies, RNA interference, and natural products, such as genistein and curcumin, have been proposed for inhibiting Notch. Currently, several classes of Notch pathway inhibitors, targeting different components of the pathway with various mechanisms of action, are not only under development, but are in clinical trials. The most promising and widely-tested manner of inhibiting Notch signaling is through γ-Secretase Inhibitors (GSIs), as the first class of Notch inhibitors to enter clinical testing for cancer [76]. Again, before Notch becomes competent for signaling, it is processed by two important enzymes, furin-like activity and γ-secretase [24]. Thus, in theory, any manipulation that interferes with Notch processing in adult tissue should impair signaling by this pathway. Originally, the idea of generating GSI was derived from the Alzheimer's field [77]. Multiple GSIs have been in various phases of clinical trials for PDAC patients, including BMS-906024, PF-030840, MK-0752, and RO4929097 [76]; however, few are being used as single agents in these trials. For example, patients with previously treated metastatic PDAC were entered into a two-stage, single-arm Phase II trial for RO4929097, an oral GSI [78]. In this study, 25% (3 of 12) evaluable patients achieved stable disease, but further enrollment during stage 2 was suspended due to the sponsor's discontinuation of RO4929097. Preclinical studies with PF-03084014 found greater efficacy in PDAC to induce apoptosis, as well as inhibit tumor cell proliferation and angiogenesis, which resulted in a reduction not only in primary tumor growth but also in metastatic dissemination, compared to gemcitabine alone [79]. According to ClinicalTrials.gov, however, any trials with PF-03084014 have been terminated or withdrawn due to change in strategy of the development of this drug. A Phase I/IIa trial has been completed for MK-0752, a potent non-competitive oral GSI, in combination with gemcitabine for the treatment of patients with surgically unresectable stage III/IV PDAC [80]. Results presented at the 2016 EORTC-NCI-AACR International Conference on Molecular Targets and Cancer Therapeutics from a phase I study indicated that LY3039478, also a GSI, is modestly effective against a range of advanced or metastatic cancers [81]. Similarly, a first-in-human phase I trial of the GSI LY900009 demonstrated the drug was tolerable, with no unexpected safety concerns and rapidly absorbed, but antitumor activity was limited [82]. None of the enrolled patients presented with a complete or partial response. While 5 of 35 patients (14%) demonstrated stable disease, these tumor types included papillary adenocarcinoma, non-small-cell lung carcinoma, ureter carcinoma, rectal carcinoma, and leiomyosarcoma, and not the 3 cases of pancreatic cancer enrolled in the study. Overall, the dose-limiting toxicity of GSI use in humans has been primarily due to secretory diarrhea, which is likely from goblet-cell

metaplasia of the small-intestinal epithelium as a result of Notch1 and Notch2 inhibition [80]. Other side effects have included skin disorders, such as erythema, rash, and pruritus, additional gastrointestinal toxicities to cause nausea and vomiting, fatigue, hypophosphatemia, and headache [80]. In order to limit these toxic side effects, regimens that employ intermittent GSI administration, such as 3-days-on-4-days-off or once a week, have been investigated based on the pharmacokinetics of specific drugs [76]. GSIs, as part of potential PDAC therapies, possess advantages that involve cost efficiency, simple administration, pan-Notch inhibition, and generally favorable tissue penetration [83, 84]. However, systemic toxicity and off-target effects remain as drawbacks. GSIs have the potential to inhibit additional γ-secretase substrates, which are more than 90 in addition to Notch receptors, and, thus, must be taken into consideration as studies evolve. The regimens for GSIs will have to be managed appropriately to not only most effectively reduce toxicity but, equally important, maintain the beneficial therapeutic effect on tumors and CSCs.

Another class of Notch pathway inhibitors that are actively under clinical development is of the monoclonal antibody (mAb) type, against either Notch receptors or Notch ligands. OMP-59R5, also known as tarextumab, is a human antibody against Notch2 and Notch3, which demonstrated reduced growth of PDAC patient-derived xenografts in combination with gemcitabine plus nab-paclitaxel [85]. In April 2016, a Phase Ib/II study of OMP-59R5 was completed in combination with nab-paclitaxel and gemcitabine in untreated patients with metastatic PDAC (ClinicalTrials.gov). Results from the phase Ib trial indicated that a 15-mg/kg dose of tarextumab combined with standard doses of gemcitabine and nab-paclitaxel was well tolerated with significant activity, in particular in patients with high Notch3 expressing tumors [86]. Another humanized mAb, OMP-21M18 or demcizumab, is against the ligand, DLL4, to block its interaction with Notch1 and Notch4. OMP-21M18 is being tested in combination with standard-of-care gemcitabine as a phase Ib trial in patients with advanced pancreatic cancer [84]. Thus far, fatigue and hypertension seem to be the most common drug-related toxicities, as reported by a phase Ib trial in patients with non-small cell lung cancer [87]. The optimism for this type of agent (mAbs) is the potential to reduce or eliminate some of the toxicities associated with GSI-based pan-Notch inhibition.

Other classes of agents under investigation to target the Notch pathway that are worth mention include "stapled peptides," decoys, and natural compounds. Stapling is a key technique for stabilizing peptides in an α-helical structure, which gives rise to a stapled peptide that is able to compete efficiently to interfere with protein–protein interactions that are mediated by α-helices. Such as the interaction of MAML with the Notch intracellular domain. A synthetic, cell-permeable, stabilized α-helical, hydrocarbon-stapled peptide derived from MAML1 has been produced, called SAHM1, which demonstrated the ability to directly bind preassembled Notch1–CSL complexes and competitively inhibit MAML1 coactivator binding [88]. Peptides such as SAHM1 have several advantages, including relatively small size, high structural compatibility with target proteins, and the ability to disrupt specific protein–protein interfaces [83]. Their utility in humans will depend on their pharmacokinetics. Decoys are soluble forms of the extracellular domain of Notch

receptors or Notch ligands, which then act as a "decoy" to compete with their endogenous cell surface-bound counterparts and eliminate Notch signaling. Although decoys of the Notch pathway have not been used in the context of pancreatic cancer yet, a Notch1 decoy has been reported that functions as ligand-dependent Notch antagonist to reduce Notch1 activity and interfere with Dll1, Dll4, and Jagged1 activities to effectively block Notch signaling in endothelial cells and thereby inhibit tumor neoangiogenesis and growth [89]. Notch signaling has also been effectively inhibited through soluble forms of the DSL type ligands Dll1 and Jagged1 [83]. Pharmacokinetics and biodistribution will be key aspects of the potential efficacy of these decoys as therapeutic options. Finally, several natural compounds appear to target Notch signaling. As mentioned earlier, genistein, which is found in soy products, inhibits Notch signaling, decreases cell proliferation, and induces apoptosis in PDAC cells, which is mediated by downregulation of NF-κB activity [75]. Sulforaphane, a natural compound derived from cruciferous vegetables, has been found to reduce the growth of CSC-xenografts derived from pancreatic tumors [90]. Similarly, quercetin, which is a major polyphenol and flavonoid commonly found in many fruits and vegetables, also inhibited growth of CSC-enriched xenografts and prevented expression of proteins involved in the EMT phenotype [91]. Certainly, the favorable concept of natural products is their relatively low toxicity, and persistent, limited Notch inhibition by natural products could be attractive for potential chemoprevention.

Although the majority of therapies against the Notch pathway are of the GSI type, the utility of others, including mAbs, stapled peptides, decoys, and natural products, warrant further investigation. Since the Notch pathway is integral for embryonic and CSC pathways, biology will be essential for managing the development of Notch inhibitors. Furthermore, monitoring Notch activity and its inhibition through biomarkers will be beneficial for measuring successful targeting. The potential challenges, however, do not outweigh the remarkable therapeutic promise offered by a pathway that is critical for proliferation and survival of cancer cells, angiogenesis, and CSC maintenance.

Conclusion

Over 100 years since the discovery of the Notch pathway, evidence has mounted in the past couple decades to implicate Notch signaling in cancer, maintenance of CSCs, and angiogenesis in a context-dependent manner. Studies initiated in model organisms such as the fruit fly provided a detailed understanding on how this pathway works at the biochemical level. Notch signaling has been associated with both normal morphogenesis and neoplastic transformation. Complementary studies in zebrafish and mice have revealed the significant relevance of this pathway in normal pancreatic morphogenesis, as well as pancreatic cancer. Moreover, alterations in this pathway have been detected in human tissue. Thus, together, these studies place Notch signaling at the center of the signaling cascades that are important to study in the pancreas and have rendered the Notch pathway an attractive

target for therapy. Importantly, developmental pathways, such as Notch, typically function together with other pathways to direct cell fate. Therefore, the most scientifically sound approach to target this pathway would be to develop mechanism-based combinations. The scientific community has reached a point where the Notch signaling pathway is largely understood at the basic molecular level. Although evidence has existed for almost two decades that Notch signaling plays a key role in cancer, there remains much to investigate. With the recent and ongoing development of agents to effectively target this pathway, this field will only continue to grow, and the promise of drugs or drug combinations that can specifically modify Notch signaling, while avoiding harmful side effects and improving both survival and quality of life for PDAC patients, remains extraordinary.

Box 1 Key Research Points

- Notch signaling is a master regulator of embryonic development in many cells and organisms. It is involved in the process of lateral inhibition where cell-to-cell contact between a signaling and a receiving cell determine fate outcome. In the exocrine pancreas, Notch is involved in acinar cell differentiation.
- Notch signaling interacts with key exocrine pancreatic transcription factors, like PTF-1, thus providing at least one mechanism by which this pathway specifies cell fate in this organ.
- Alterations in Notch signaling are a cause of several diseases, including certain malignancies. Notch is altered during the metaplastic progression that leads to pancreatic cancer and ductal adenocarcinoma. These findings make Notch a potential therapeutic target for therapeutic interventions.

Box 2 Future Scientific Directions

- Notch is involved in both pancreatic morphogenesis and pancreatic cancer. Fortunately, different types of animal models and model organisms exist to better understand the mechanism by which this pathway instructs these processes.
- Studies on crosstalk between Notch signaling with other cascades in pancreatic cells is very well-established for a few pathways. Therefore, expanding this area of research will provide a better understanding of pancreatic physiology and pathobiology.
- Historically, some of the knowledge on the Notch pathway that has been derived from studies in nonpancreatic cell systems has been directly applied to normal and neoplastic pancreatic cell biology. However, recent studies, which indicate that well-known mediators of the Notch pathway regulate pancreatic morphogenesis in a Notch independent manner, require a careful extrapolation of data from the literature and in depth molecular experimentation in the pancreas itself.

Box 3 Clinical Implications

- Fortunately, prototype drugs have been derived from the knowledge gained on the biochemistry of Notch signaling. Currently, several classes of Notch pathway inhibitors, which target different components of the pathway by various mechanisms of action, are not only under development but are in clinical trials. These

include several protease inhibitors, in particular, the γ-secretase inhibitors (GSIs), which have been the first class of Notch inhibitors to enter the clinic.

- GSIs, which are the major tool for manipulating Notch signaling, are among the most advanced drugs. However, these drugs are not very specific, as γ-secretase cleaves numerous substrates besides Notch. Therefore, side effects are common. The development of additional types of Notch pathway inhibitors, including monoclonal antibodies (mAbs), stapled peptides, and decoys, offers promise to diminish side effects and improve the therapeutic index.
- Since Notch deregulation appears to already occur at the preneoplastic stage (PanINs) and these lesions are very frequent in normal and pancreatitis patients, it remains to be explored if natural compounds that target this pathway, such as genistein, sulforaphane, and quercetin, are beneficial for the chemoprevention of pancreatic cancer.

Cross-References

- ▶ Developmental Molecular Biology of the Pancreas
- ▶ Diagnostic Biomarkers
- ▶ Emerging Therapeutic Targets in Pancreatic Adenocarcinoma
- ▶ Hedgehog Signaling Plays a Dual Role in Pancreatic Carcinogenesis
- ▶ Pancreatic Cancer Stem Cells
- ▶ Smad4-TGF-β Signaling Pathways in Pancreatic Cancer Pathogenesis
- ▶ The Molecular Pathology of Precursor Lesions of Pancreatic Cancer

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Stromal Inflammation in Pancreatic Cancer: Mechanisms and Translational Applications

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Contents

Abstract

Pancreatic ductal adenocarcinoma is the most severe form of pancreatic cancer because of pronounced inflammation and desmoplasia leading to hypoxia, metabolic reprogramming, and immune suppression that ultimately promote tumor

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growth and metastasis. The conventional wisdom is that patient survival is hobbled by the inability of currently available therapies to penetrate the tumor and its dense stromal microenvironment. The pancreatic cancer stromal microenvironment is a heterogeneous population of cancer cells, immune cells, cancer-associated fibroblasts, vascular endothelial cells, and neurons. While a detailed understanding of the cells, mediators, and receptors influencing stromal dynamism continues to emerge, interactions between these cells influence tumor suppression as well as tumor promotion. The specific roles for the inflamed stroma in pancreatic cancer immune evasion, progression, metastasis, and therapeutic resistance likely depend on stage of tumor development and distinct biophysical features within the dynamic cellular micro-niches of the tumor. Uncovering the stromal mechanisms of tumor development and progression should prompt the discovery of key windows of opportunity for multimodal therapies in pancreatic cancer.

Keywords
Inflammation · Stellate Cell · Cytokine · Desmoplasia · Cancer-Associated Fibroblast · T Cell · Tumor-Associated Macrophage · Immune Evasion · Stromal Remodeling

Introduction

The most common form of pancreatic cancer, pancreatic ductal adenocarcinoma (PDA), originates from epithelial cells lining the exocrine ducts of the pancreas. Despite Herculean efforts and numerous diverse clinical trials, death rates from PDA remain nearly equal to incidence rates. This is largely due to the relatively unique biology of PDA, namely, the dissemination of tumor cells to distant sites (liver, peritoneum, lung) very early following malignant transformation at the primary site in the pancreas. Therefore, despite tremendous advances in therapeutic opportunities, durable disease control (cure) remains elusive even though survival durations have increased with the application of more effective multimodality therapy [1]. However, overall survival time remains inferior to other solid tumors, and a major reason for this is the inaccessibility of currently available therapies to penetrate the tumor and its dense stromal microenvironment. PDA, relative to other solid tumor malignancies, is characterized by a prominent desmoplasia, with 80–90% of the tumor parenchyma comprised of dense fibrotic stroma enveloping the cancer cells (Fig. 1).The intense fibrosis of the PDA stroma results in pronounced tumor hypoxia and a unique form of hypovascularity that restricts the effectiveness of radiotherapy and impedes the delivery of chemotherapeutic drugs [2, 3]. Thus, therapies to specifically target the stroma were implemented in an effort to sensitize PDA to radiation and chemotherapy. However, clinical trials with stromal-targeted therapies have shown minimal efficacy [4]. Concomitant with those results from the clinic, accumulating evidence from preclinical PDA animal models revealed that

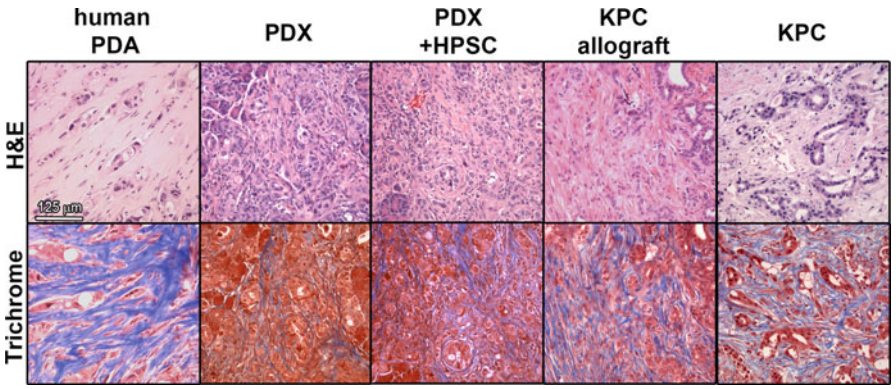

Fig. 1 Histopathology of the stromal compartment in human and murine models of pancreatic cancer. Photomicrographs of hematoxylin and eosin (H&E; *top*) or Masson's trichrome (*bottom*) stained clinical and preclinical primary exocrine pancreatic ductal adenocarcinoma (PDA) tumor tissue. Staining reveals abundant remodeling and deposition of collagen (*blue trichrome staining*) in human patient tumors (PDA) and the genetically engineered KPC mouse model. Primary tumors from xenografted patient-derived cells (PDX) show little collagen deposition or fibrosis. Mixing patient-derived cells with human pancreatic stellate cells (HPSC) leads to slightly more collagen and stromal remodeling. Allografting murine KPC PDA cancer cells into syngeneic mice resulted in higher levels of fibrosis relative to the PDX immune-incompetent model systems

complete ablation of the cellular stromal compartment, surprisingly, led to even more aggressive tumor biology [5, 6]. These findings suggest the stroma is a dynamic tissue whose effects on tumor progression cannot simply be viewed as positive or negative. Cumulatively, the emerging literature indicates that a more nuanced clinical and research approach is required to better understand the role of the stroma in the development, progression, and therapeutic resistance of PDA.

PDA develops through the dysregulation of cancer cell-autonomous and non-cancer cell-autonomous signaling pathways that parallel defined morphological changes in the pancreas. These structural modifications within the pancreas arise from acinar-to-ductal metaplasia that progress into pancreatic intraepithelial neoplasia (PanIN 1–3) leading to invasive carcinoma [7]. PanIN formation is characterized by changes in ductal architecture as well as an influx of innate myeloid and adaptive lymphoid immune cells [8]. The predominant molecular changes within the ductal epithelium are activating mutations in KRas, a critical event found in >90% of PDA patients. Subsequent changes in tumor suppressor genes, p16 and p53, and Smad4, also known as Deleted in Pancreatic Cancer-4 (DPC4), are associated with the transformation of ductal epithelial cells into invasive carcinoma. These cancer cell-autonomous changes parallel profound remodeling of the stromal matrix surrounding the transformed epithelium. Desmoplasia, the deposition and/or remodeling of connective tissue, around the malignant duct in PDA is the result of a fibrotic stromal reaction by diverse fibroblasts and immune cells and their secreted products. The cellular constituents of the stroma consist of pancreatic stellate cells (PSC),

fibroblasts, vascular endothelial cells, immune cells, and cancer cells. Cells within the tumor parenchyma also produce a dynamic array of acellular components such as collagen, fibronectin, hyaluronan, and other glycosaminoglycans, as well as cytokines, growth factors, and proteases that lead to the production and deposition of new extracellular matrix. Ultimately, these molecular constituents establish the biophysical properties of PDA tumors thought to be critically important to the characteristic aggressive biology associated with disease.

Within the normal pancreas, resident fibroblasts, PSCs, and their associated connective tissue components, along with leukocytes, and vascular endothelial cells act to homeostatically repair tissue and coordinate wound repair. Akin to many mucosal tissues, during pancreatic injury or tissue damage, ductal epithelial cells upregulate a pro-inflammatory gene program resulting in the secretion of cytokines, growth factors, and proteases, as well as production of reactive oxygen and nitrogen species [9]. These soluble mediators impact the diverse array of cells present within the pancreatic mucosa. The cumulative effect of this inflammatory influx is to synthesize and remodel the extracellular matrix, neovascularize the tissue, coordinate normal innate and adaptive immune surveillance mechanisms, and stimulate epithelial wound closure to repair the injured pancreas. However, in the setting of premalignant and malignant tissue, transformed epithelial cells bearing oncogenic mutations alter the normal wound repair processes ultimately resulting in the desmoplasia and stromal remodeling indicative of PDA. The extreme concentration of cellular infiltrates together with the presence of acellular components establishes an immune repressive, hypoxic, nutrient-deprived, and avascular microenvironment novel among solid tumors.

Inflammation and Activated Stromal Cells

In normal wound healing, activated fibroblasts play key roles in the secretion of cytokines and chemokines, recruitment of immune cells, and the deposition and remodeling of the extracellular matrix (ECM). Cancer-associated fibroblasts (CAFs) constitute the cellular majority within the evolving PanIN to invasive PDA carcinoma. CAFs synthesize and secrete extracellular matrix proteins, participate in the recruitment of suppressive leukocytes, and stimulate the proliferation and dissemination of transformed cancer epithelial cells. CAFs are activated fibroblasts derived predominantly from resident quiescent PSCs but can evolve from resident fibroblasts, bone marrow-derived mesenchymal cells, and/or the epithelial-to-mesenchymal transition (EMT) of normal epithelial cells (Fig. 2). The primary identifiable cellular marker for CAFs is α-smooth muscle actin (α-SMA), a cytoskeletal protein closely associated with smooth muscle cells which led to their further classification as myofibroblasts. Additional identifiers of CAFs include fibroblast activation protein, fibroblast specific protein 1, vimentin, and platelet-derived growth factor (PDGF) receptors. This repertoire of CAF markers reflects the diversity and heterogeneity of these cells, a finding that is only beginning to be understood [10]. CAFs dynamically secrete a broad array of molecules to contribute to and remodel the

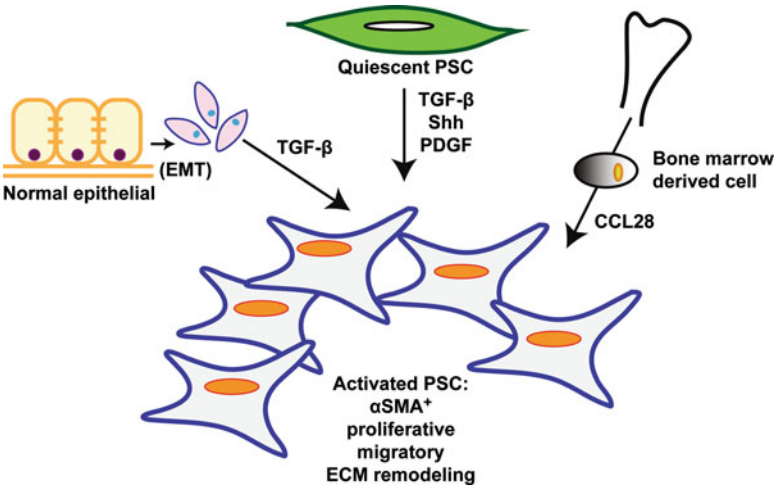

Fig. 2 Derivation of cancer-associated fibroblasts in pancreatic cancer. Activated pancreatic stellate cells (PSC) are abundant and drive much of the stromal remodeling and deposition in pancreatic ductal adenocarcinoma (PDA). Activated PSCs are characterized by elevated α-smooth muscle actin (α-SMA) expression, as well as proliferative, migratory, and enhanced ECM-producing capacity. Increased levels of activated PSCs are thought to result from transforming growth factor (TGF)-β, Sonic hedgehog (Shh), and platelet-derived growth factor (PDGF) stimulation of quiescent PSC present within the normal pancreas. Recent reports suggest activated PSC levels reflect recruitment of mesenchymal bone marrow-derived stem cells, perhaps through inflammatory chemokines such as CCL28 produced by cancer epithelial cells. Activated PSC may also be derived from a subpopulation of epithelial cells that have undergone TGF-β-mediated epithelial-to-mesenchymal transition (EMT)

ECM. Further, CAFs contribute to cytokine, chemokine, and growth factor production in the inflammatory stromal reaction that ultimately influence the establishment of the immunosuppressive PDA milieu (Fig. 3) [11].

The major source of CAFs in the PDA stroma is the resident quiescent PSC. In the normal pancreas, quiescent PSCs make up approximately 4% of the total cellular composition of the organ distributed in connective tissues and localized predominantly near ducts, blood vessels, nerves, and pancreatic lobules [12]. Their function, under homeostatic conditions, is thought to be related to the storage of vitamin A and the production of proteases needed to remodel the mucosa and submucosa of the exocrine pancreas. During tumorigenesis, progression from quiescent to activated PSCs stimulates the loss of vitamin A containing lipid vacuoles, induces expression of α-SMA (myofibroblast-like), and increases PSC proliferation and migration. The precise mechanism(s) behind the conversion from quiescent to activated PSC remains poorly understood. The prevailing theory is that the transition is triggered by environmental cues, largely inflammatory in nature, such as alcohol and its metabolites, reactive oxygen species produced during oxidative stress, as well as the release of transforming growth factor-β (TGF-β), PDGF, or Sonic hedgehog by epithelia cancer cells [13].

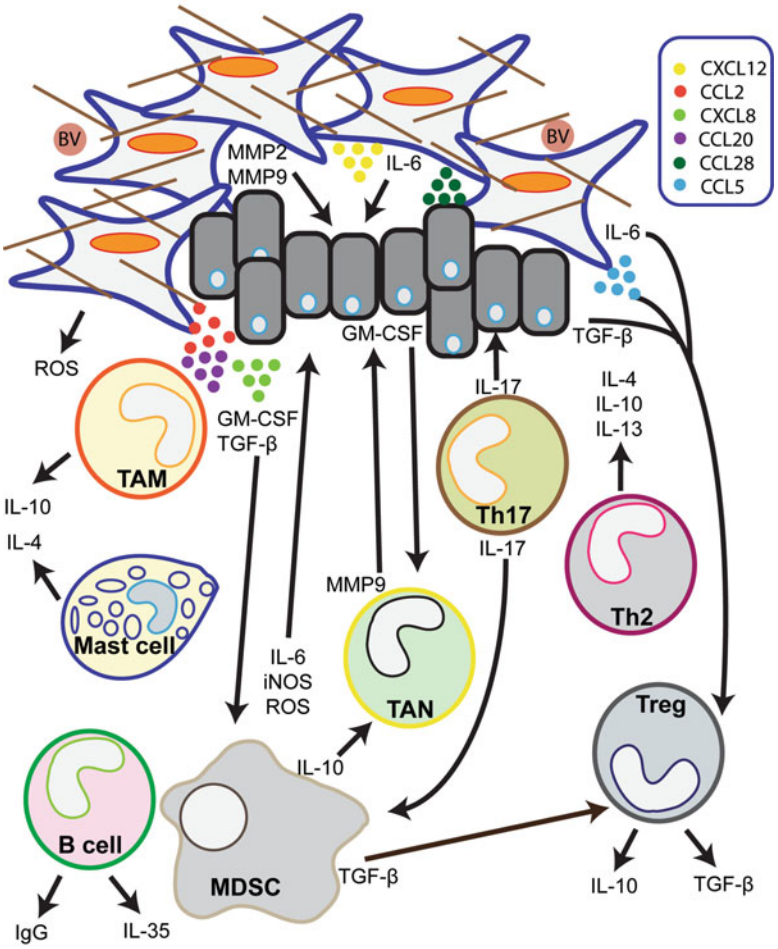

Fig. 3 Stromal inflammatory cells and mediators in the pancreatic cancer microenvironment. The complex tumor microenvironment of pancreatic ductal adenocarcinoma (PDA) reflects a collection of activated pancreatic stellate cells (PSC), blood vessels (BV), and innate and adaptive immune cells surrounding the transformed cancer epithelial cells. Secreted mediators produced by these varying cell types influence the recruitment and activation of tumor-associated macrophages (TAM), tumor-associated neutrophils (TAN), mast cells, B cells, and T helper cells (Th2 and Th17) as well as immune-suppressive myeloid-derived suppressor cells (MDSC) and regulatory T cells (Treg)

In addition to quiescent PSCs, bone marrow-derived mesenchymal cells may comprise a precursor cellular source for CAFs. Several groups have employed sex-mismatched murine transplantation/cell reseeding models to demonstrate that tagged mesenchymal stem cells reseeded to recipient mice expand and contribute to both the quiescent and activated PSC/CAF populations, as marked by the expression of desmin and α-SMA. While these bone marrow-derived mesenchymal cells appear

to be in the minority of the total tumor stromal cell population (<5%), their presence and function require further examination as they may have cell-specific spatial and/or temporal functions in the inflamed stroma.

During EMT, transformed epithelial cells of the exocrine duct lose expression of epithelial markers, notably β-catenin and E-cadherin, while acquiring expression of the mesenchymal markers vimentin and N-cadherin. The cumulative effects of the genetic and epigenetic changes leading to EMT of PDA cells result in the transition from stable epithelial cell-cell junctions typical of a normal exocrine duct to more non-adherent transformed cells with mesenchymal properties. Coculturing tumor epithelial cells with activated PSCs resulted in epithelial cell acquisition of fibroblast markers and fibroblast morphology in response to TGF-β produced by activated PSCs/CAFs. Thus, tumor epithelial cells may acquire phenotypic features and functional properties of CAFs during the EMT process that disrupt the homeostatic host defense immune response and contribute to tumorigenesis.

Inflammation and Stromal Immune Cells

Leukocytes, as well as surveilling lymphocytes, residing within the normal exocrine pancreas mucosa protect the organ from infectious agents and remove and repair damaged epithelium. These cells are actively antitumor early in the development of PDA. However, over time within the hypoxic fibrotic tumor mass encasing the transformed epithelium, these leukocytes differentiate into the more immune-suppressive tumor-promoting subsets. Thus, immunologically, the developing tumor shifts from a protective inflammatory environment into a non-protective immune evading milieu that suppresses both innate and adaptive arms of the immune system. The convergence of immunosuppressive M2-type macrophages, N2-type neutrophils, regulatory T cells, as well as myeloid-derived suppressor cells cumulatively dampens the ability of tumor-reactive cytotoxic T lymphocytes or helper T cells to remove the malignant cancer cells. The sum effect of this transition from pro-inflammatory to immune evasion is enhanced desmoplasia and the development of invasive carcinoma (Fig. 3).

Macrophages are vital innate immune responders that phagocytose dead or dying cells, facilitate wound healing, and regulate tissue homeostasis. Macrophages can differentiate into distinct lineages in response to stimuli within the surrounding tissue microenvironment. The M1 lineage is predominantly associated with pro-inflammatory host defense, while the M2 lineage is more closely involved with immune dampening. Monocytes recruited to, and that differentiate within, the tumor mass are most often the immunosuppressive M2 tumor-associated macrophage (TAM). The combination of the oncogenic KRas driver mutation within the transformed duct cells, with the secretion of inflammatory cytokines and growth factors by PSCs in the inflamed stroma, notably, TGF-β,interleukin (IL)-4, IL-10, IL-13, and epidermal growth factor (EGF), participates in TAM reprogramming into the M2 lineage. Chemokines, namely, CCL5 and CCL20, direct the recruitment, trafficking, and spatial organization of TAMs within the developing tumor, while

activated integrins and focal adhesion kinase signaling coordinate TAM adherence to stromal matrix proteins. M2-TAMs have multiple, wide-ranging effects in the stromal microenvironment, which cumulatively function to establish and maintain immune dampening and fibrosis via secretion of numerous immunosuppressive mediators, notably TGF-β, inducible nitric oxide synthase, arginase-1, indoleamine 2,3-dioxygenase, and the cytokine IL-10. M2-TAMs in the inflamed microenvironment promote aspects of tissue remodeling and wound healing. In particular, M2-TAMs aid in the digestion of extracellular matrix through production of matrix metalloproteinases (MMPs) and promote angiogenesis via vascular endothelial growth factor (VEGF) production [14]. Analysis of human PDA tissues revealed a correlation between high levels of infiltrating M2-TAMs, identified using the markers CD68, CD163, and CD204, with an increase in lymph node metastasis. TAMs also secrete cytidine deaminase, a pyrimidine salvaging enzyme capable of digesting and inactivating gemcitabine, contributing to immune-mediated chemotherapy resistance. The M1/M2 lineage model provides a useful, if simplified, framework to consider macrophage functions in tumorigenesis. However, there is increasing appreciation that macrophages exist in a continuum of phenotypes, fulfilling distinct functional roles in the inflamed stroma of solid tumors. In total, TAMs modify nearly every aspect of a tumor's development, from cancer cell proliferation and motility to invasiveness, angiogenesis, immunosuppression, extracellular matrix reorganization, and treatment resistance.

While M2-TAMs have a critical role in the development of PDA and the initial establishment of immune suppression, a separate monocyte lineage, the myeloid-derived suppressor cells (MDSC), appears to be a key contributor of the immune constraining microenvironment in late stages of tumor progression. MDSCs potently inhibit T cell proliferation, migration, and effector lymphocyte functions, blunt the antitumor cytotoxic effects of Natural Killer (NK) cells, and expand the suppressive regulatory T cell population. MDSCs originate from bone marrow hematopoietic stem cells and are a heterogeneous population of immature immune cells with angiogenic and immune-suppressive functions [15]. They can be derived from either a monocytic, m-MDSC, precursor lineage, or a granulocytic, gr-MDSC, precursor lineage. The chemokines CCL2 and CCL5 recruit newly derived MDSCs from the bone marrow to the established tumor. Secretion of various cellular mediators from M2-TAMs (arginase), CAFs (IL-6), and cancer cells (GM-CSF) play a pivotal role in MDSC expansion and migration which cumulatively promote continued immunosuppression in the tumor microenvironment [16]. Clinically, high levels of MDSCs are associated with reduced overall survival.

In addition to these myeloid innate immune cells, granulocytic cells also play a role in stromal inflammation in PDA. While levels of circulating neutrophils are elevated in patients with PDA, significant numbers of infiltrating neutrophils are uncommon within the tumor itself. Despite limited numbers infiltrating the tumor, a poor clinical outcome is correlated with increased levels of neutrophils in the tumor tissue in comparison with adjacent pancreas, supporting prior reports indicating neutrophils play a role in inflammation-driven tumorigenesis [17]. Tumor-associated neutrophils (TANs) arise from two lineages: N2-type neutrophils are

pro-tumorigenic and result from high localized concentrations of TGF-β, while N1-type neutrophils which are anti-tumorigenic and stem from elevated levels of IFN-γ. The pro-neoplastic phenotype of N2-TANs reflects their contribution to ECM degradation, promotion of neovascularization, and immunosuppression in the developing tumor. Infiltration of TANs into the tumor is largely influenced by CXCL8 and CXCL16 chemokine gradients produced by tumor epithelial cells. These same neutrophil populations secrete other chemokines, including CCL2, CCL3, CCL19, and CCL20, promoting recruitment of monocytes and dendritic cells to the tumor. TANs also participate in the inflammatory reaction and stromal remodeling by secreting a variety of pro-tumorigenic factors, including IL-2, IL-6, IL-10, TNF, and VEGF, matrix remodeling serine-proteases MMP-8 and MMP-9, and production of reactive oxygen species (ROS).

Mast cells are a myeloid granulocyte traditionally associated with allergy and anaphylactic reactions. However, mast cell numbers have been shown to be elevated in PDA and correlated with the presence of metastatic disease, higher tumor grade, and worse prognosis [18, 19]. Accumulating evidence supports a role for mast cells as tumor-promoting immune cells. Intratumoral mast cells were observed in genetically engineered mouse models of pancreas cancer, consistent with observations in human tissues. Mast cell secreted IL-13 promoted PSC proliferation and TGF-β expression, while conditioned medium from mast cells also stimulated growth of PDA cell lines. Additional studies in mast cell-deficient mice demonstrate reduced tumor growth. PSCs produce IL-33, a known pro-inflammatory molecule and activator of mast cells. Inhibition of the chemokine receptor CXCR4 using an FDA-approved small molecule antagonist blocked mast cell migration into PDA primary tumors and limited tumor expansion in a syngeneic mouse allograft model. In contrast, a newly described inducible genetically engineered mouse model suggests mast cells possess minimal, if any, effect on PDA progression [20]. Thus, more research is needed to clarify mast cell involvement in the cellular and extracellular dynamics of the stromal and immune microenvironment in PDA.

While innate immune cell subsets are critically important in inflammatory host defense responses, T lymphocytes of the adaptive immune system play roles in removal of cell-associated antigens or pathogens and are vital in the killing of cancer cells. T cell infiltrates, detected using the general T cell marker CD3, have been reported in both human and murine PDA tissue [21]. $CD3^+$ T cells can be further subdivided into cytotoxic T lymphocytes that form the first line of defense against tumors, as well as helper T cells which support antitumor responses. Natural killer cells (NK cells) are a separate subclass of lymphocytes, distinct from T or B cells, which play key roles in innate immune responses to lyse microbially infected cells or cancer cells. NK cells also produce the cytokine IFN-γ and can thus participate in the adaptive immune responses. There are three predominant helper T lymphocyte subtypes, each identifiable by the expression of the CD4 coreceptor and further defined by the type of cytokines produced. Helper T cells (Th) are classified as either Th1 cells that produce IFN-γ; Th2 cells secreting IL-4, IL-5, IL-13, and IL-10; or Th17 cells which secrete IL-17 and IL-22 cytokines. Th1 cells promote inflammation and participate in host defense to bacterial and viral pathogens. Th2 cells

contribute to allergic hypersensitivity and participate in host defense against extracellular antigens or helminth parasites. Th2 cells also negatively regulate Th1 cells by inhibiting their effector functions. Th17 cells function prominently at mucosal surfaces and trigger pro-inflammatory danger signals to promote neutrophil mobilization and the expression of host defense mediators. IL-17 and IL-22 secreted by Th17 cells promote NF-κB-dependent and JAK/STAT3 transcription factor signaling, respectively, which promote the early inflammatory cascade, and bridge the innate and adaptive immune processes.Th2 and Th17 cells secrete cytokines that stimulate a tumor-favorable, growth-enhancing, microenvironment. In contrast, $CD8^+$cytotoxic T cell functions are supported by $CD4^+$ Th1 cells through the production of proliferative IL-2 and regulatory IFN-γ cytokines histopathologically correlated with small PDA tumors. The functional role of $CD4^+$ T cell subsets was examined employing genetically engineered mouse models that express KRas in a tissue-specific and inducible manner [22, 23]. For example, when an inducible KRas murine PDA model was crossed with CD4 knockout mice, inflammation-associated tumor progression was abrogated [23]. The inability to establish a tumor-promoting environment in the absence of $CD4^+$ cells was attributed to the increased number of tumor-infiltrating antitumor $CD8^+$ T cells. In another approach, an inducible PDA/pancreatitis model resulted in pro-inflammatory Th17 cell recruitment to the tumor that synergistically increased tumor progression, suggesting that pro-inflammatory helper T cell subsets promote PDA development.

Regulatory T cells (Treg), defined by cell surface and cytoplasmic $CD4^+$, $FoxP3^+$, and $CD25^{high}$ markers, are critical effectors of peripheral immune tolerance, suppressing effector T cells through the secretion of the immune dampening mediators IL-10 and TGF-β and cell surface receptors CTLA-4 and PD-1 [24]. The combination of these secreted cytokines and receptor proteins potently inhibits the antitumor functions of $CD4^+$ Th1 cells and $CD8^+$ cytotoxic T cells, as well as NK cells. Accordingly, given the immune suppression in PDA, there is an elevated number of Treg cells in the tumor that is correlated with poor patient survival [25]. Infiltration of Treg cells into the desmoplastic stroma is mediated by activated PSCs and cancer epithelial cell secretion of chemokines such as CCL5, as well as altered expression of adhesion molecules on tumor-associated vascular endothelial cells. Further, TGF-β produced by CAFs can initiate the conversion of conventional effector $CD4^+$ T cells into a population of "induced" Treg cells [26, 27]. Depletion of Tregs increased the levels of tumor-reactive T cells and was correlated with smaller murine PDA tumors. Information from clinical trials indicate that gemcitabine, a common chemotherapeutic in PDA, reduced levels of gr-MDSC and Treg cells and was correlated with improved number of effector $CD8^+$ effector T cells in patients.

While evidence for humoral immunity in PDA has been relatively scarce, emerging data implicate B cells in important roles within the stromal environment of pancreatic cancer. Histopathologic analyses indicate B cells localized in proximity to neoplastic regions in both murine and human pancreatic cancer [28]. PDA murine models revealed that B cells were recruited to the tumor microenvironment via the chemokine CXCL13, which was itself produced and secreted by activated PSCs.

Once within the tumor microenvironment, B cells appear to play a pro-tumorigenic role through the secretion of IL-35, a positive effector of tumor cell proliferation. A pro-tumorigenic role for B cells was also identified through their production of IgG, which led to macrophage reprogramming into M2-TAMs through FcγR signaling. Pancreas-specific knockout of the hypoxia-inducible factor (HIF)-1α transcription factor revealed a significant increase in effector B cells into the pancreas and exacerbation of disease in a murine model. Treatment of HIF-1α-deficient mice with a B cell-depleting anti-CD20 monoclonal antibody relieved PanIN progression associated with more aggressive disease phenotype, suggesting humoral immunity has a role early in PDA development. Although roles for key factors in the inflammatory stroma have been implicated in B cell tumor responses, roles for helper T cells in the development of humoral tumor immunity, or B cell involvement in immune suppression or epithelial transformation and development and progression from PanIN to PDA, have yet to be defined.

In sum, the creation of a tumor-permissive environment results from an imbalance of antitumor versus pro-tumor immune cell populations (Fig. 3). The cells that are notably absent from pancreatic tumors include immune effector cells such as $CD8^+$ T cells, dendritic cells, and NK cells, which are actively excluded from the tumor by suppressor factors within the microenvironment. Similarly, the balance of $CD4^+$ helper T cells is skewed to have an increased proportion of immune-suppressive Th2 with a minimal infiltration of the Th1 effector arm. Coincident with these changes is the elevation in Treg cells that dampen the antitumor effector functions of activated lymphocytes and whose trafficking is regulated by other chemoattractants.

Inflammatory Mediators

Communication within the normal exocrine pancreas mucosa is mediated by an array of cytokines and growth factors produced by non-transformed and transformed epithelial cells, resident fibroblasts, and quiescent PSCs, together with macrophages and neutrophils of the innate immune response. Communication with the PDA microenvironment is bidirectional as transformed pancreatic cancer cells produce pro-inflammatory mediators, immunoregulatory cytokines and chemokines, as well as growth factors that act in a paracrine fashion on PSCs, CAFs, and leukocytes. Growth factor secretion by CAFs is indispensable in promoting tumor progression and metastasis. The resultant genetic and epigenetic changes evoked by cytokine and growth factor signaling within the tumor microenvironment act to increase cancer cell proliferation, mobility and dissemination, as well as activation and reprogramming of CAFs, TAMs, and T cells.

High serum levels of the pro-inflammatory cytokine IL-6 have been reported in patients with PDA and shown to promote a tumor-associated inflammatory environment in murine models [29, 30]. Activated PSC/CAFs are the primary source of pro-inflammatory IL-6. Secreted IL-6 binds to and triggers the IL-6 receptor, a member of the class I cytokine receptor family, to activate STAT3. The STAT3

signaling pathway has been implicated in several key aspects of PDA progression. Initial reports demonstrated roles for IL-6-mediated differentiation of peripheral naive $CD4^+$ T helper cells into Th17 cells and expansion of MDSCs [22]. IL-6 may also drive EMT of pancreatic cancer cells, suggesting a nonimmune pro-tumorigenic role for this pro-inflammatory molecule. IL-6 activation of STAT3 in pancreatic cancer cells may also upregulate DNA methyltransferase enzymes that participate in epigenetic changes in the tumor, perhaps silencing key genes involved in malignant progression [31, 32]. Thus, there is a well-documented role for IL-6 in PDA progression, proliferation, migration, and angiogenesis. In agreement with these broad effects, inhibition of IL-6 or STAT3 signaling has been shown to blunt tumor progression in preclinical models of pancreatic cancer.

Production of IL-17 and IL-22 by Th17 cells mediates pro-inflammatory host defense responses to extracellular pathogens and repair of mucosal tissues. IL-17 binds to the IL-17 receptor, a class I cytokine receptor that signals through the TRAF6 and NF-κB transcription factors. The resultant functional effects of IL-17 signaling are the synergistic expansion of TNF and IL-1 cytokine effects to recruit monocytes and neutrophils to the site of inflammation. IL-17 is also involved in the progression of PanINs and their transition to invasive pancreatic cancer [22]. Consistent with the plasticity of helper T cell subsets, IL-17 appears to be pro-tumorigenic, particularly early in tumor development. Anti-tumorigenic effects of Th17 cells appear later in tumor progression, with cells restricted to the peripheral margins of the established tumor [33]. Understanding the role of IL-17 in the progression of PDA was facilitated by genetic overexpression or genetic depletion studies in murine model systems. Overexpression of IL-17 accelerated PanIN development and progression, while loss of IL-17 was associated with decreased MDSC infiltration, perhaps reflecting reduced myelopoietic GM-CSF cytokine levels, as well as decreased IL-6 production. Further, the effects of IL-17 were not restricted to immune cells, as signaling through the IL-17 receptor expressed on KRas-activated epithelial cells was shown to promote carcinogenesis.

Isoforms of the pro-inflammatory IL-1 cytokine are elevated in PDA and appear to have discrete effects on tumor progression. IL-1 is the first of a large and expanding family of cytokines with distinct, cell-type-specific pro- or anti-inflammatory properties. Originally named lymphocyte-activating factor and subsequently identified as IL-1, it has mitogenic and pyrogenic properties and is among the earliest mediators of an inflammatory response. Within the malignant pancreas, high IL-1α levels are associated with poor patient prognosis [34, 35]. IL-1α produced by CAFs and TAMs regulates integrin expression impacting the development and migration potential of invasive cancer epithelial cells. IL-1β produced by CAFs and leukocytes influences macrophage recruitment, CAF activation, and the promotion of metastasis. Cell culture experiments and PDA murine models demonstrated that IL-1's biological effects signal through its canonical activation of the NF-κB transcription factor, which can be abrogated therapeutically using the anakinra IL-1 receptor antagonist.

Tumor necrosis factor (TNF), a master regulator of inflammation produced by macrophages and stromal fibroblasts, was originally called cachectin and

characterized as a regulator of myeloid antitumor cytotoxicity [36]. However, in a departure from its originally defined antitumor properties, TNF within the developing PDA tumor drives the production of numerous other cytokines and chemokines. This cytokine storm aids in establishing the immune-suppressive microenvironment and promoting tumor proliferation and migration. Indeed, in mouse models, overexpression of TNF correlates with progression from PanINs to invasive carcinoma. As expected from a cytokine with pleiotropic effects, TNF influences expression of mediators of hedgehog signaling, which plays a key role in stromal matrix remodeling, and the NF-κB-dependent upregulation of chemokine such as CCL2, CXCL1, and CXCL8 that drive macrophage and neutrophil trafficking and tumor infiltration. TNF may therefore play a key role in establishing or expanding the early inflammatory microenvironment. Over longer periods of time and as concentrations remain elevated, TNF's biological effects in established late-stage malignant PDA tumors likely reflect its more systemic roles in cachexia.

Reactive oxygen species (ROS) are free radicals produced during oxidative stress that cause damage to lipids, proteins, and DNA. Oxidative stress is characterized by a shift in the equilibrium between ROS levels and antioxidant compounds that mitigate its effects. Interestingly, cancer cells have adapted to tonically maintain levels of ROS at a lower threshold in order to avoid cell death. In PDA, oncogenic KRas-induced expression of both NADPH-oxidase stimulated formation of ROS and upregulated levels of detoxifying antioxidant signaling pathways. The concomitant production of ROS with antioxidant molecules acts to maintain a tonic level of pro-tumorigenic ROS which ultimately promotes tumor progression through a combination of signaling pathways in cancer cells [37]. As ROS is membrane diffusible, it may also act as a mediator with effects on CAF or immune cells within the tumor microenvironment. In the developing PDA tumor, ROS is also produced by monocytes in response to pro-inflammatory cytokine signaling. Leukocyte-produced ROS participates in the activation of quiescent PSCs, an effect exacerbated by epithelial PDGF. CAF-produced ROS has been shown to induce the polarization of monocytes into M2-TAMs, demonstrating that cancer cells are not the sole source of ROS in the PDA tumor microenvironment. It is therefore likely that ROS production by epithelial cells, as well as the surrounding stromal and immune cells within the inflamed tumor, cumulatively amplifies tumorigenic stimuli and promotes stromal desmoplasia.

TGF-β plays a complicated role in directing the epithelial cancer cell-autonomous, tumor microenvironmental, and systemic responses that cumulatively regulate the initiation, progression, and malignancy of numerous human cancers. TGF-β has a similarly complicated regulatory role in the human immune system. This complexity holds true in PDA, with TGF-β and its receptor-mediated activation of Smad4 playing a major role in the stromal inflammatory microenvironment and tumorigenesis. TGF-β serves as a tumor suppressor early in tumor development and progression inhibiting epithelial cell proliferation and accelerating apoptosis of metaplastic cells. However, elevated levels of TGF-β increasingly promote tumorigenesis through cancer epithelial cell migration and invasion, angiogenesis, as well as suppression of the antitumor immune system in later stages of tumor progression.

TGF-β is secreted by a collection of cells including macrophages, lymphocytes, CAFs/PSCs, epithelial cells, and platelets. Within the developing pancreatic tumor, TGF-β produced by cancer cells and PSCs stimulates EMT of ductal epithelia, VEGF-mediated neovascularization, and PSC activation and inhibits cytotoxic $CD8^+$ T cells, M1 macrophages, dendritic cells, and NK cells. Normally, TGF-β signaling through Smad4 promotes the induction of angiogenesis and immune suppression; however, this tumor-suppressive function is lost in PDA upon the inactivation/loss of *Smad4/Dpc4* by cancer epithelial cells [38]. Smad4 is a signal transducer activated by TGF-β that regulates expression of integrins, E-cadherin, and collagen, all of which are repressed in over 50% of invasive pancreatic adenocarcinomas. Genetically engineered mouse models have established the critical role for TGF-β in the development and exacerbation of PDA. When combined with oncogenic KRas mice, *Smad4* deletion, or knockout of the receptor, *Tgfbr2*, activated by the cytokine, accelerated the progression of KRas-initiated tumors. These in vivo data suggest that Smad4 mediates the tumor inhibitory action of TGF-β signaling at early stages of tumor development. Consistent with the pleiotropic nature of the cytokine, increased TGF-β levels as well as Smad4 loss/inactivation are associated with poor prognosis in patients.

IL-10 is an immunosuppressive cytokine overrepresented in tissue and serum from patients with unresectable PDA. Within the PDA tumor microenvironment, IL-10 is largely produced by Th2 cells, Tregs, and M2-TAMs. IL-10-mediated immune suppression within the pancreatic cancer tumor microenvironment correlates with reduced antitumor NK cell functions, reduced dendritic cell activity, as well as a demonstrative shift toward Th2 cell cytokine production. Consistent with its inhibitory effects, IL-10 also limits expansion and functional effects of Th1 cells. Histopathological analyses of human PDA specimens indicate IL-10 is produced by M2-TAMs located at the tumor periphery, which would be expected to limit entry and antitumor effects of cytotoxic T cells.

Chemokines, or chemotactic cytokines, represent a large family of more than 50 secreted proteins with a wide range of function in normal physiology. Chemokine functions include direction of immune cell trafficking, angiogenesis, and wound healing. As secreted molecules, chemokines travel in the circulation, diffuse through the parenchyma and extracellular matrix of tissues, and bind and activate their cognate receptors expressed on target cells. Much like other cytokines, chemokine expression is highly regulated during specific disease states. A variety of chemokines have been linked, either through histopathologic analyses of human specimens or using mouse models, with stromal inflammation and biologic effects on pancreatic cancer cells.

CXCL12, originally termed stromal-derived factor 1 based on its discovery in bone marrow stromal cells, and its cognate receptor CXCR4 are homeostatic chemokines with key roles in lymphocyte circulation. Data from knockout animals indicate the requirement of CXCL12 and CXCR4 signaling in neural development, vasculogenesis and lymphopoiesis/myelopoiesis. Conventional wisdom has been that the metastatic homing of cancer cells reflects CXCL12 produced by distant target tissues [39, 40]. However, CAFs are a tumor-proximal source of CXCL12 as epithelial expression of the *Cxcl12* gene is epigenetically repressed in transformed

PDA cancer cells [40]. Genetic reexpression of *Cxcl12* in pancreatic cancer cells reduced their proliferation and migration, suggesting that CXCL12 may have tumor-suppressive properties [40]. Subsequent reports demonstrate key antitumor effects of recombinant CXCL12 administered as a biologic therapy, with decreased metastasis and growth of primary tumors [31, 41]. The discordant effects of CXCL12 resulted in part from the concentration-dependent oligomerization of the chemokine ligand, with native protein or engineered monomeric variants providing a pro-tumorigenic signal, while elevated wild-type chemokine or mutant dimerized protein repressed tumor progression [31, 42]. The biologic effects of CXCL12 are mediated by CXCR4 whose expression is elevated in PDA and participates in cancer cell proliferation and migration [40]. CXCR4 is also expressed by the innate and adaptive immune cells localized within and surrounding the tumor. Combined chemotherapy to block immune suppression in conjunction with CXCR4 receptor antagonists blunted PDA growth in a preclinical mouse model and led subsequently to a clinical trial [NCT02179970] [43]. Stromal CXCL12 may influence the spatial organization and retention of $CXCR4^{+}$cytotoxic $CD8^{+}$ T cells in the juxta-tumoral stromal compartments, preventing access of these effector cells into the tumor mass.

CCL2 is a key regulator of monocyte/macrophage infiltration into the pancreatic cancer tumor via the engagement of the receptor CCR2. CCL2 secretion by pancreatic tumor cells is amplified in response to pro-inflammatory IFN-γ, TNF, and IL-1β cytokine stimulation [44]. Further, consistent with tissue damage and inflammation, CCL2 levels were markedly elevated following radiotherapy. CCL2 and CCR2 levels are enhanced in tumor tissue from an orthotopic murine pancreatic cancer model that was established to mimic the inflammatory milieu of human disease. Elevated serum levels of CCL2 in patients with pancreatic cancer correlated with a poor prognosis. A phase 1b single-center, open-label, non-randomized clinical trial was performed to judge the efficacy of supplementing FOLFIRINOX chemotherapy with the CCR2 inhibitor PF-04136309 [45]. While efficacy data is not yet available, the results of this study suggest that the combination treatment of the CCR2 inhibitor with FOLFIRINOX is safe and tolerable.

CXCR2 is activated by the ELR-motif CXC chemokines CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8 to promote the chemotaxis and bone marrow mobilization of neutrophils and gr-MDSC to sites of inflammation in the tumor [46]. CXCR2 signaling is upregulated in both MDSCs and TANs within the neoplastic pancreas, while tumor cells and CAFs have limited CXCR2 expression. In the PDA murine model, gr-MDSCs and neutrophils are the most prominent CXCR2-expressing cells [46]. Genetic or immune depletion of CXCR2 in the PDA murine model revealed a role for CXCR2 in distal metastasis, whereas growth and proliferation of the primary tumor were slowed but not prevented. In agreement with its expression by gr-MDSC, the loss or inhibition of CXCR2 greatly improved T cell infiltration into the tumor, and the combination of CXCR2 inhibitors with PD1 checkpoint blockade significantly extended survival.

The inflammatory chemokine CCL20 mediates its effects through its sole cognate receptor CCR6. As in colon cancer, both CCL20 and CCR6 are overrepresented in pancreatic tumor compared to normal pancreas [47]. In addition, M2-TAMs produce

CCL20 to functionally mediate the chemotactic migration and invasion of $CCR6^+$ tumor cells. Both cell culture and preclinical mouse model systems demonstrated the role for CCL20 and CCR6 in promoting tumor cell proliferation and migration in vitro and growth and metastasis to the liver in vivo. Consistent with reports implicating CCR6 in the pathogenesis of autoimmune psoriasis and colitis, pro-inflammatory Th17 cells infiltrate the tumor in response to PDA CCL20 production. Thus, CCL20 likely has an underappreciated role in pancreatic cancer.

Trafficking of lymphocytes and dendritic cells into lymph nodes is controlled largely through the chemokine receptor CCR7 [48]. Stimulation of CCR7-expressing PDA tumor cells by the chemokine CCL19 upregulates expression of the transcription factor Twist, which, by signaling through ERK and PI3K/AKT, facilitates EMT of cancer cells. CCR7-expressing pancreatic cancer stemlike cells expressing CD133 were also responsive to CCL21, which stimulated migration, survival, and EMT. Consistent with its known role in lymphoid tissue trafficking of immune cells, expression of CCR7 by pancreatic cancer cells resulted in an increased frequency of metastatic tumor cells within lymph nodes [49]. Intratumoral injection of CCL21 abrogated tumor progression by blunting lymph node trafficking of pancreatic cancer cells and was associated with higher numbers of tumor-reactive T cells in the primary tumor.

The chemokine CCL5, previously termed RANTES, coordinates the recruitment of CCR1-, CCR3-, and/or CCR5-expressing monocytes and T cells [50]. Pancreatic cancer cells that produce elevated levels of CCL5 actively recruit $FoxP3^+$ Tregs to the tumor microenvironment. Notably, interruption of CCL5 signaling reduced Treg levels within the tumor coincident with a decrease in primary tumor size. Another potential source of CCL5 is mesenchymal-derived stromal cells. These stromal cells have been shown to upregulate CCL5 when cocultured with cancer epithelial cells through engagement of the insulin-like growth factor (IGF) with its cognate receptor.

The chemokine CCL28 is an established mediator of mucosal-directed immune cell trafficking. CCL28 signaling through its cognate receptor, CCR10, promote migration of activated PSCs [51]. An RT-PCR screen of chemokine receptors uncovered the abundant expression of CCR10 and its ligand CCL28 in PDA cell lines. Immunohistochemical analyses of human primary PDA tissues revealed the expression of the ligand was restricted to cancer cells, while the receptor was abundant on both quiescent and activated PSCs as well as cancer epithelial cells. Inflammatory conditions upregulated the expression of CCL28 by pancreatic cancer cells and mediated migration of CCR10-expressing PSCs without altering their activation state. Thus, pro-inflammatory cancer epithelial cell-produced CCL28 chemokine may influence recruitment and localization of newly activated PSCs or bone marrow-derived mesenchymal cells to the PDA stroma.

Inflammation and Matrix Components

The extracellular matrix (ECM) is an essential noncellular component of all tissues and organs. The ECM serves not only as a molecular scaffold to organize soluble constituents but also acts as a biochemical and biomechanical mediator of tissue

morphogenesis, differentiation, and homeostasis. The role of the ECM in the stromal microenvironment is to provide crucial interactions that guide tumorigenesis, cell migration, invasion, and metastasis [52]. Although the ECM is composed primarily of water, proteins, proteoglycans, and polysaccharides, it is dynamic and heterogeneous and is constantly under a state of remodeling due to nonenzymatic and enzymatic modifications. Tumor cells and CAFs actively contribute to the remodeling of the ECM via an array of growth factors, such as fibroblast growth factor-2, TGF-β, and PDGF, influential proteolytic enzymes, and by de novo matrix or glycosaminoglycan deposition [12]. Activated PSCs secrete an array of ECM proteins that ultimately form the signature fibrotic scar seen histologically in PDA. The accumulation and elevation of these connective tissue components are posited to distort the normal ductal architecture leading to a compression of vascularization, poor transvascular permeability, and, in turn, hypoxia within the tumor [53, 54]. The fibrosis and stromal remodeling characteristic of PDA exacerbates tumor progression and renders the tumor resistant to chemotherapy and radiologic intervention.

The PDA stroma is enriched with various glycoproteins including collagens, fibronectin, and tenascin C, the clear majority of which is produced and deposited by activated PSC/CAFs within the tumor. While the primary collagens associated with PDA are types I and III, CAFs in culture may also produce collagen types I, III, IV, and V. Collagen provides tensile strength and rigidity to the tumor and contributes to the chemotactic migration of cancer epithelial cells, PSC/CAFs, and leukocytes. By exerting tension on the matrix, fibroblasts organize and align collagen fibrils into sheets and cables. Normal quiescent fibroblasts in the pancreas secrete collagen in a random isotropic manner, whereas an organized anisotropic arrangement of relatively straight collagen fibers is indicative of tumor-associated desmoplasia. Anisotropic collagen patterning can serve as a highway for cancer epithelial cell migration during invasion and metastasis. CAF-produced fibronectin is intimately involved in directing the organization of the interstitial ECM and, like collagen, has a crucial role in mediating cancer epithelial cell attachment, migration, and tumor metastasis. Consistent with EMT in many solid tumors, there are shifts in the matrix constituents, with elevated fibronectin and collagen I levels observed in human PDA tissue specimens. Genetically engineered mouse models of PDA constructed to selectively abolish α-SMA-expressing CAFs abrogated collagen and fibronectin deposition and stromal remodeling in PDA tumors [5]. Depletion of α-SMA myofibroblasts and their associated soluble mediators early during PanIN formation, or later in overt PDA, resulted in the development of significantly more invasive, undifferentiated, and hypoxic tumors compared to control mice, suggesting that stromal CAFs and/or the desmoplasia and inflammatory factors they produced beneficially curtailed tumor progression.

Sonic hedgehog (Shh), a member of the hedgehog family of genes involved in mammalian organogenesis, is aberrantly overexpressed in 70% of human PDA tissue specimens [55]. Shh binding to and activation of its receptor, Patched, which is expressed on CAFs, stimulate the membrane translocation of the Smoothened signaling protein, which subsequently activates downstream signaling pathways regulating gene expression. Activation of Smoothened target transcription

factors such as Gli led to changes in the pancreatic extracellular matrix and cytokine release, including Wnt and insulin-like growth factor (IGF), which promoted tumor growth [56]. Shh produced by tumor epithelial cells may function through autocrine signaling, regulating cancer epithelial cell proliferation and differentiation, and may function in a paracrine manner on neighboring PSC/CAFs to mediate pancreatic fibrosis. Co-activation of Shh and oncogenic KRas in a transgenic mouse model rapidly induced PanIN formation and shortened survival of tumor-bearing mice. A preclinical study using a Smoothened inhibitor, in combination with gemcitabine, markedly improved vascularization of PDA and survival in a preclinical model [53]. However, clinical trials using a commercially available hedgehog inhibitor were unsuccessful, with phase II studies showing limited benefit or, in some instances, being terminated early due to increased mortality [57]. Similarly, deletion of Shh from the stromal environment using genetically engineered mouse models resulted in cancers that were more aggressive, more proliferative, and presented with reorganized stroma notable for its increased vasculature [6]. The therapeutic efficacy of this genetic approach was replicated using an anti-stromal chemotherapy approach using a hedgehog inhibitor [58]. Evaluation of three genetically modified mouse strains further confirmed that inhibiting hedgehog signaling accelerates tumor progression. Suppressing stromal desmoplasia accelerated growth of the PanIN epithelium, while hedgehog activation caused stromal hyperplasia and reduced epithelial proliferation. Taken together, there is accumulating evidence from clinical trials and preclinical models supporting key protective roles for the stroma in PDA progression.

Mucins are a large family of high molecular weight O-glycosylated polypeptides typically expressed by epithelia cells to maintain and protect the normal pancreas mucosa from invading pathogens. Based on their diverse physiological and structural characteristics, mucins have been classified into a transmembrane subfamily and a secreted subfamily. Secreted mucins form the protective mucus layer on the apical surface of mucosal epithelia of the gastrointestinal, respiratory, and reproductive tracts. While the normal exocrine pancreas expresses low levels of MUC1, the expression of both transmembrane (MUC1, MUC3, MUC4, MUC7, MUC13, MUC16, and MUC17) and secretory mucins (MUC5AC, MUC5B, and MUC6) is aberrantly overexpressed in PanIN and PDA and has been linked to disease progression, poor prognosis, and chemoresistance. This likely reflects the ability of mucins to contribute to the immune-suppressive environment, alter signaling through receptor tyrosine kinases directly on cancer cells, or regulate cancer cell detachment, invasion, and metastasis [59]. Tumor epithelia are not the sole producers of mucins as MUC1 expression by Treg cells enhances their proliferation and cytokine production. Pancreatic cancer cells may become cross-linked to M2-TAMs or dendritic cells via mucins leading to increased production of immune-suppressive IL-10 and decreased secretion of the T cell chemoattractant CCL3. Given their aberrant expression early in tumor development, mucins have made an attractive target for diagnosis. The mucin polypeptide backbone is predominantly coated with O-linked carbohydrates. The sialyl Lewis carbohydrate CA19–9 prevalent on MUC1 is the most common FDA-approved prognostic marker for pancreatic cancer, but it can

also be elevated in colon and biliary cancers, when liver function is abnormal, and in a variety of benign conditions especially involving the lungs. In addition, Lewis antigen-negative patients do not produce CA19–9 and will not demonstrate marker elevation regardless of the extent of disease. At present, there is no reliable blood test for the early diagnosis of pancreatic cancer in asymptomatic individuals without a high-risk syndrome.

Hyaluronan (hyaluronic acid) is a large linear anionic non-sulfated glycosaminoglycan that retains water to provide elasticity to connective tissue. Under normal conditions hyaluronan is a key constituent in epithelial wound repair, inflammation, angiogenesis, and immune, epithelial, and fibroblast cell migration. Hyaluronan levels are tightly balanced by controlling its synthesis (hyaluronan synthases) and degradation (hyaluronidases). Hyaluronan is physiologically increased during an inflammatory response, likely in response to cytokines such as IL-1 and TNF upregulating hyaluronan synthase enzymes. Extracellular hyaluronan binds to its major receptor CD44 expressed largely on lymphocytes with a subset expressed on pancreatic cancer cells [60, 61]. In PDA, hyaluronan levels can be overabundant and correlate with increased tumor growth and migration. Abundance of hyaluronan is thought to be a major factor in PDA chemoresistance since it is the main contributor to the elevated interstitial fluid pressure, vascular collapse, and decreased vascular permeability associated with impaired drug delivery. However, hyaluronan signaling through CD44 promotes immune-suppressive signaling, M2-TAM reprogramming, and Treg localization, suggesting additional roles in tumorigenesis by modulating the immune microenvironment of PDA. De-bulking the tumor stroma by enzymatic digestion of hyaluronan was tested in preclinical models using PEGylated hyaluronidase PH20 (PEGPH20). Treatment of established murine PDA tumors with PEGPH20 relieved the elevated interstitial fluid pressure and re-expanded the stromal microvasculature of the tumor, ultimately sensitizing the cancer cells to gemcitabine treatment and extending overall survival [54]. Based on the strength of these preclinical studies, PEGPH20, in combination with chemotherapeutic interventions, has advanced to clinical trials [NCT01453153; NCT01839487; NCT01959139] and showed promise for improved drug efficacy, especially in those patients whose tumors demonstrate a greater level of staining (percent of the tumor tissue) with a hyaluronidase-binding protein [62].

Periostin and syndecans are additional ECM proteins or heparan-sulfate proteoglycans, respectively, with roles in PDA stromal remodeling and disease progression. Periostin, a CAF-secreted ECM protein, has been shown to be upregulated in PDA tissue compared to normal pancreas and was correlated with poor patient survival. Elevated levels of periostin can participate in a feedback loop that increases CAF fibrogenic activity while supporting tumor growth under serum and oxygen starvation characteristic of PDA tumors. Periostin binds to integrins on tumor cells activating EGFR-mediated intracellular signaling pathways, inducing EMT, and increasing tumor cell survival, invasion, and metastasis. Syndecan-1 and syndecan-2 have been shown to be upregulated in pancreatic cancer. The role for syndecans in PDA metastasis, especially related to tumor growth and movement along perineural fibers, has been observed [63].

Matrix metalloproteases (MMP) are members of a large family of calcium-dependent zinc-containing enzymes responsible for degrading and organizing the ECM. MMPs act on a variety of structural ECM components including collagens, fibronectin, and tenascin C enriched in PDA tumors. Immunohistochemical analysis of human PDA specimens revealed elevation of MMP-2, MMP-7, and MMP-9 primarily by cancer epithelial cells. Genetic ablation of epithelial MMP-9 resulted in increased levels of IL-6 production which subsequently promotes tumor cell growth and metastasis through the activation of STAT3 signaling in cancer epithelial cells [64]. Thus, MMP-9 may function as a tumor suppressor. Conversely, MMP-7 may act as a tumor-promoting factor since its downregulation abrogated PDA cancer cell proliferation [65]. Indeed, MMP-7 was shown to activate Notch signaling and, in turn, the dedifferentiation of exocrine duct epithelial cells. Notch signaling is a key mechanism of tumor progression that could be targeted therapeutically using γ-secretase inhibitors. MMPs also inactivate chemokines through amino-terminal proteolysis and may therefore play a role in sculpting chemokine inflammatory communication within the tumor microenvironment.

Tissue inhibitors of metalloproteases (TIMP) are the natural inhibitors of MMPs, and the balance between MMP and TIMP expression is an important variable in metastatic tumor progression. TIMP sterically and reversibly bind to the MMP catalytic zinc domain in a 1:1 stoichiometric ratio that, if unbalanced, can greatly influence the composition of the ECM within the tumor microenvironment. Cancer epithelial cell TIMP1 expression and secretion are increased in human tumor specimens and in tissues from PDA mouse models. However, it is still unknown if elevated levels of TIMP1 are a secondary response to increased tumor cell-derived MMPs or if TIMP1 itself is pro-tumorigenic. Taken together, a collection of differentially expressed proteolytically active MMPs and inhibitory TIMP enzymes contribute to tumor development and progression through their ability to selectively degrade components of the extracellular matrix that surrounds PDA.

Dynamic Inflammatory Stroma Milieu

These cellular and acellular mediators communicate in a dynamic interplay in the tumor microenvironment of preneoplastic and metaplastic PDA lesions (Fig. 4). The precise role(s) of the unique PDA cells and matrix stromal components in tumor progression and treatment remains incompletely understood but is an active area of research. The conventional wisdom that the stroma has tumor-promoting, tumor-protective, roles is increasingly in flux, with a tumor-suppressive, host-protective, role for the inflammatory stroma emerging. Indeed, complete ablation of desmoplastic stroma has been viewed as a therapeutic approach to limit tumor growth. Paradoxically, evidence from mouse models indicates that complete ablation of the stroma results in tumors that became more aggressive with an accelerated rate of tumorigenesis. By contrast, the idea of chronically "normalizing" activated stroma by reprogramming desmoplasia from a tumor-promoting to a tumor-restrictive state has been suggested to hold therapeutic promise [66, 67]. The identification of a

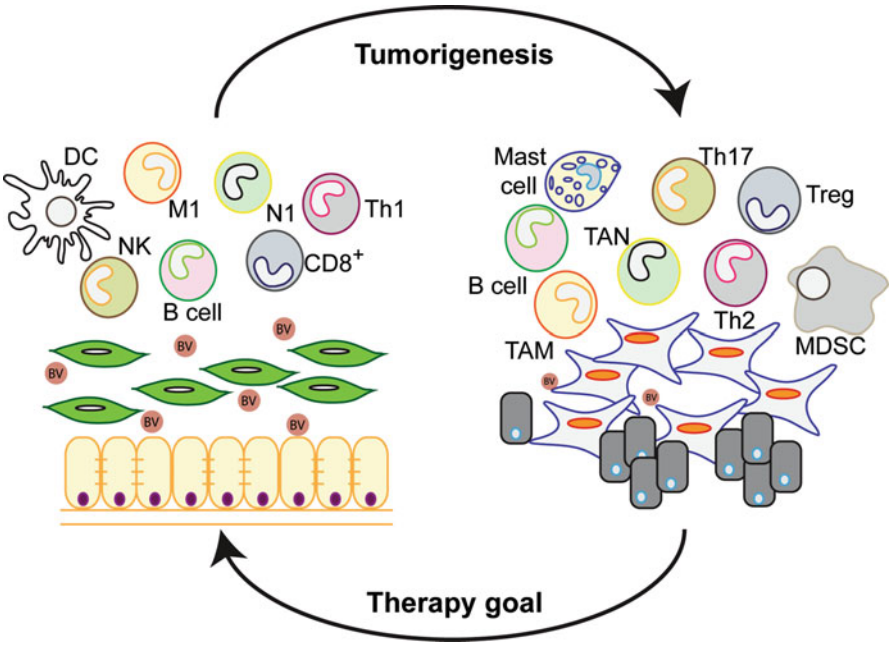

Fig. 4 Schematic overview of the dynamic balance between stromal and cancer cells within the pancreatic cancer milieu. In the normal pancreas (*left*) resident fibroblasts, quiescent pancreatic stellate cells (*green*), leukocytes, and vascular endothelial cells (BV) reside in close proximity to an intact ductal epithelium. Collectively these cells, and their mediators, coordinate wound repair and promote host defense to pathogens. Pancreatic injury or tissue damage upregulates a pro-inflammatory gene program that following oncogenic epithelial duct transformation (*right*) results in the secretion of cytokines, growth factors, reactive oxygen and nitrogen species, and proteases that remodel the stroma, reprogram and suppress immune cells, and play an essential role to promote tumor formation, growth, progression, and metastasis

clinically applicable way to revert desmoplastic stroma to normal is of considerable interest. To better categorize the heterogeneous PDA stroma, image analysis software to facilitate histopathology assessment of the discrete localization of various stromal markers has been developed [68]. Additional exploration of the secretome and cell surface marker expression profiles illustrated that CAFs are a highly heterogeneous population of cells that is made up of several subpopulations that are differentially regulated both spatially and temporally. Thus, it is possible that the conflicting reports on the tumor-promoting or tumor-suppressive properties of the inflamed stroma reflect the cellular heterogeneity within the microenvironment, with each of the varying components playing key temporal roles in tumor formation and malignant progression.

The fibrotic PDA stroma directs the formation of a hypovascular and hypoxic microenvironment, both of which likely contribute to the failure of anti-angiogenic, antiproliferative, and, to some degree, radiation therapies. In response to tissue hypoxia, pancreatic cancer cells and PSCs potently increase their expression of the

transcription factor HIF-1α [69]. The constriction and restriction of vascular growth in the PDA stroma are a conundrum in that uncontrolled tumor cell growth often requires an increased demand for oxygen and nutrients, a need that is usually compensated for through angiogenesis. Human PDA tissues are poorly vascularized and have fewer larger diameter (>10 μm) blood vessels compared to normal exocrine pancreas [53, 54]. While activated PSCs are more potent than pancreatic cancer cells in secreting pro-angiogenic substances such as VEGF in culture, both cell types exert an anti-angiogenic phenotype in the hypoxic tumor through the sustained and elevated deposition of new ECM proteins and secretion of cytokines and growth factors. Similarly, angiogenic factors produced by PSCs resulted in localized foci of angiogenesis localized to the tumor periphery. Given that hypoxia is typically a late event in tumor development, the cytokines produced likely provide positive feedback that exacerbates the remodeling and fibrosis initiated in the earliest stages of metaplasia, subsequently counteracting the pro-angiogenic factors.

Poor diffusion of oxygen and nutrients as well as blood vessel constriction by the dense stroma within pancreatic tumors results in cancer cell metabolic reprogramming in favor of glycolysis (Warburg effect) and activation of pro-survival stress responses. In fact, poor perfusion, hypoxia, and accompanying metabolic changes have been correlated with tumor aggressiveness [70]. Possible mechanisms for metabolic reprogramming in PDA during microenvironmental stress include those that are HIF-1α dependent and/or selection for mutations in oncogenes and tumor suppressors. Glucose deprivation also promotes KRas mutation in tumors, presumably by creating a selective pressure for such genetic aberrations [71]. Conversely, oncogenic KRas signaling can drive expression of GLUT1 glucose transporter and/or other metabolic factors that contribute to the reprogramming of bioenergetic metabolism.

Pro-survival stress responses including the integrated stress response and endoplasmic reticular stress (ER stress) response, also known as the unfolded protein response, are also induced by chemotherapy in PDA. This can lead to resistance to genotoxic tumor killing [72]. Induction of key regulators of the unfolded protein response such as GRP78 participated in resistance to chemotherapeutic agents in PDA by activating survival signaling factors including Akt. CRR9, a surface-expressed protein induced by ER stress and elevated in tumors, encoded by the cancer susceptibility gene candidate CLPTM1L, promoted pancreatic tumor cell survival under ER stress as well as chemoresistance [73]. CRR9 may exert this function through interaction with GRP78 at the plasma membrane and mediation of downstream survival signaling. Other tumor cell survival proteins such as dual-specificity phosphatase 1 are activated by oxidative, hypoxic, metabolic, and chemotherapeutic stresses resulting in chemoresistance.

The induction of autophagy in response to metabolic stresses is well known. Autophagy, "self-eating," appears to be an underlying stress-induced mechanism of PDA cancer epithelial cell survival, including that induced by chemotherapy [74]. Additional evidence suggests that autophagy may provide a pool of chemotherapeutic resistant quiescent cancer stem cells capable of becoming reactivated and in turn facilitating disease recurrence [75]. High expression of

autophagy and cancer stem cell markers was identified on human pancreatic tumors and was associated with poor survival. Further, autophagy appears to be upregulated in activated PSCs, with autophagy inhibitors inducing lipid droplet acquisition coincident with decreased IL-6 secretion and ECM production. While there is some debate on whether enhancement of autophagy-mediated killing or autophagy inhibition therapy should be pursued, emerging data support a role for autophagy cancer stemness, tumor progression, chemoresistance, and poor clinical outcome in patients with PDA.

Conclusions

The tumor microenvironment is a dynamic three-dimensional structure that supports epithelial ductal carcinoma formation and propagation through an altered extracellular matrix and is maintained by diffusible paracrine growth factors and cytokines. The inflammatory stroma in pancreatic cancer is a heterogeneous population of cancer cells, immunocytes, CAFs, vascular endothelial cells, and, as increasingly recognized, unmyelinated neurons. Cross talk between cells present within the tumor microenvironment plays an essential role in the development of an environment to promote tumor formation, growth, progression, and metastasis. The interaction between cells through direct contact or the release of cytokines, growth factors, and chemokines acting in an autocrine and/or paracrine fashion plays an essential role in controlling tumor growth. Increasingly it is recognized that the stromal microenvironment has tumor-suppressive as well as tumor-promoting properties. The distinct roles for the stroma likely depend on stage of tumor development, localization within the overall tumor mass, and distinct biophysical features within the micro-niches of the tumor. A detailed understanding of these features, including the cells, mediators, and receptors influencing stromal dynamism, continues to emerge. The sum effect of this complex stroma is that elevated chemokine production recruits inflammatory cells into the developing tumor that are ultimately reprogrammed by cytokines, growth factors, and other mediators from pro-inflammatory host defense leukocytes into immune-evading suppressor cells. Uncovering the precise target and depth of anti-stromal interventions should foster the discovery of key windows of opportunity for combinatorial therapies which may include immune checkpoint blockade, metabolic inhibitors, and cytotoxic compounds. The potential for stroma-based therapies to effectively target the tumor microenvironment and result in clinically meaningful improvements in patient survival will be based on the continued basic understanding of pancreatic cancer biology (Fig. 4).

Cross-References

► Borderline Resectable Pancreatic Cancer
► Developmental Molecular Biology of the Pancreas

- ▶ Diagnostic Biomarkers
- ▶ Emerging Therapeutic Targets in Pancreatic Adenocarcinoma
- ▶ Hedgehog Signaling Plays a Dual Role in Pancreatic Carcinogenesis
- ▶ Mouse Models of Pancreatic Exocrine Cancer
- ▶ Role of Tumor-Stromal Interactions in Pancreatic Cancer Invasion and Metastases
- ▶ Smad4-TGF-β Signaling Pathways in Pancreatic Cancer Pathogenesis
- ▶ Surgical Resection for Pancreatic Cancer Using the International Study Group of Pancreatic Surgery (ISGPS) Classifications
- ▶ The Molecular Pathology of Precursor Lesions of Pancreatic Cancer

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Mouse Models of Pancreatic Exocrine Cancer

Pedro A. Pérez-Mancera

Contents

Abstract

Pancreatic ductal adenocarcinoma (PDA) is virtually a lethal disease, with most patients dying of pancreatic cancer within one year of diagnosis. This poor prognosis, due to the innate resistance of PDA to both chemotherapy and

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radiotherapy, exists despite tremendous advances in our understanding of the molecular and cellular basis of PDA pathogenesis. Therefore, there is an urgent need to find molecular targets that can help to develop novel therapeutic approaches to improve the diagnosis and survival of PDA patients. The use of genetically engineered mouse models (GEMMs) of pancreatic cancer, as described here, have enabled a comprehensive investigation of the genetics and biology of the disease, opening new avenues to elucidate the molecular mechanisms involved in the pathogenesis of pancreatic cancer as well as the response to different therapeutic intervention strategies.

Keywords
Pancreatic cancer · Genetically engineered mouse models (GEMMs) · KRAS · Tumor genetics · Gene validation · Preclinical platform

Introduction

Pancreatic ductal adenocarcinoma (PDA) remains as an almost uniformly lethal disease with an overall 5-year survival rate of ~6% [1]. In 2012, approximately 338,000 new cases of PDA were diagnosed worldwide, and over 331,000 patients died from this disease, making it the seventh most common cause of cancer death (*GLOBOCAN, 2012*). Dismally, it is expected that PDA will become the second cause of cancer-related death by 2030 [2]. This dire clinical situation exists despite extensive efforts conducted over the last two decades to understand the genetics and biology of PDA, and is mainly due to both its early metastatic potential and its innate resistance to systemic chemotherapy and radiotherapy. This outcome highlights the urgent need to find new routes to combat PDA. Over the past three decades, the continuous improvement in gene targeting technologies has allowed the generation of refined genetically engineered mouse models (GEMMs) of pancreatic cancer that has closely mimicked the pathogenesis of the human disease. How GEMMs have supported the investigation of PDA pathogenesis, helping to unveil cancer promoting mechanisms and potential therapeutic targets, will be discussed in this chapter.

Pathogenesis of Human PDA

PDA is the most frequent and most lethal pancreatic neoplasia, representing >85% of all pancreatic neoplasias. Histologically, PDA emerges through a well-established sequence of microscopic preinvasive lesions (Pancreatic Intraepithelial Neoplasia; PanIN), associated with a relatively small number of frequently altered key genes. PanINs are classified from stages I (low grade) to III (high grade), with accumulative degrees of cytologic and architectural atypia through stages II and III. High grade PanINs eventually transform into frank PDA with areas of growth beyond the basement membrane [3, 4] (Fig. 1).

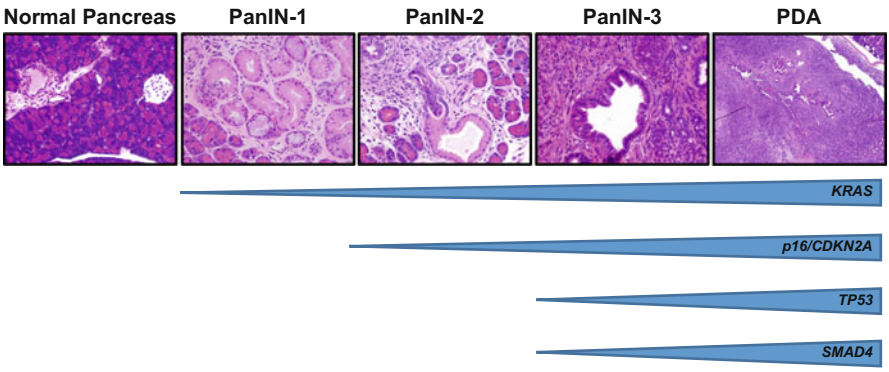

Fig. 1 PanIN to PDA progression model. Activating mutations in the oncogene KRAS are considered the initial mutational step in pancreatic cancer and induce the generation of PanIN-1 lesions. The progression from low-grade PanIN-1 to high-grade PanIN-3 lesions is associated with the accumulation of specific genetic alterations that include inactivation of *p16/CDKN2A* at an intermediate stage, and the inactivation of *TP53* or *SMAD4* at later stages

Early molecular profiling studies performed in the 1990s supported a pancreatic cancer progression model through the identification of genetic alterations that were accumulated in higher grade PanINs [5]. Activating mutations in *KRAS*, mainly at codons 12, 13, and 61, are detected in >90% of pancreatic cancer specimens [6]. Since *KRAS* mutations were detected in 36% of low grade PanINs and 87% of high grade PanINs [7], they are considered to be the initial molecular mutational step of PDA. Oncogenic RAS isoforms are refractory to GAP activity and conserve their active Ras-GTP conformation, allowing their interaction with multiple downstream effectors to trigger diverse cellular responses [8]. Among these effectors, RAF-MEK-MAPK, phosphatidylinositol 3-kinase (PI3K)-AKT, and Ral guanine nucleotide exchange factor (RalGEF) are the most extensively studied RAS effector pathways [9–11]. Oncogenic KRAS induces low-grade PanINs that progress to PDA following the acquisition of additional genetic and epigenetic alterations. Among them, the most relevant alterations include inactivation or point mutation of *p16/CDKN2A* (>95%), *TP53* (70%–75%), and the transforming growth factor (TGF)-β pathway components *DPC4/SMAD4* (55%), *TGFβRI* (<5%), and *TGFβRII* (<5%) [12–15] (Fig. 1). However, and despite massive efforts, these targets remain undruggable. Additionally, this relatively consistent mutational spectrum does not explain neither the strong resistance of PDA to chemotherapy and radiotherapy nor one of the most lethal features of PDA, the ability of the PDA tumor cell to invade the surrounding tissue and metastasize in other organs. It is hypothesized that the major molecular and cellular mechanisms involved in resistance to therapies and cellular dissemination of the PDA cell remain to be identified.

Besides PanIN lesions, the commonest pancreatic preneoplasia, two other pancreatic precursors, Intraductal Papillary Mucinous Neoplasia (IPMN) and Mucinous Cystic Neoplasia (MCN) [16] have received increasing clinical attention in the last few years. These preneoplastic pancreatic cystic lesions, less characterized

molecularly than PanINs, have the capability to progress to frank PDA. Therefore, unveiling the molecular basis underlying the development of the cystic lesions is critical for understanding the pathogenesis of pancreatic cancer. Notably, GEMMs of pancreatic cancer have accelerated our understanding of the genetic events involved in the development of the different pancreatic precursors, and in the progression of these preneoplastic lesions to invasive and metastatic PDA.

During the last decade, the extensive development of whole genome analysis approaches has confirmed the relevance of genes identified in the initial genetic studies performed in the 1990s, including KRAS, *p16/CDKN2A*, *TP53*, and *DPC4/SMAD4*. Additionally, these studies have identified a plethora of new potential key players, dramatically improving our knowledge of the genetic abnormalities that characterize PDA development and confirming that pancreatic cancers harbor a substantial genomic heterogeneity. A seminal study by Jones et al. [17] performed whole exome sequencing and copy number analyses of 24 human PDA tumors. This study identified more than 1000 somatic mutations, with an average of 63 genetic alterations per tumor. These alterations were grouped within 12 key signaling pathways that were each altered in at least 67% of the tumors, with apoptosis, KRAS signaling, G1/S regulation, Hedgehog signaling, TGFβ signaling, and Wnt/Notch signaling affected in 100% of the tumors [17].

More recently, Andrew Biankin and Sean Grimmond, employing integrated genomic approaches, have unveiled major biological pathways involved in the pathogenesis of pancreatic cancer. Using next-generation exome sequencing and single nucleotide polymorphism profiling analysis of paired normal-tumor DNA samples from 99 PDA patients, they have provided new insights into the molecular pathways dysregulated during pancreatic cancer progression. This approach identified 2,016 nonsilent mutations, with 16 genes extensively mutated, including known PDA driver genes (such as *KRAS*, *TP53*, *CDKN2A*, *SMAD4*, or *ARID1A*). Additionally, novel PDA candidate genes involved in chromatin modification (*EPC1* and *ARID2*) or DNA damage repair (*ATM*) were identified. Interestingly, this study also unveiled a critical role for axon guidance regulators (SLIT/ROBO signaling) in PDA development [18]. Importantly, two *Sleeping Beauty* transposon-mediated PDA mouse models delivered unbiased genetic evidence supporting the potential involvement of axon guidance genes in pancreatic carcinogenesis [19, 20]. These models, which will be discussed below in this chapter, have provided valuable examples of how GEMMs of pancreatic cancer can be used to understand the molecular basis of PDA pathogenesis. A subsequent analysis of 100 PDA specimens identified new candidate drivers of PDA including *KDM6A* and *PREX2*. This study also defined, based on chromosomal structural rearrangements, four subtypes of PDA with potential clinical value: stable (20% of total PDAs), locally rearranged (30%), scattered (36%), and unstable (14%). The unstable group was associated with the inactivation of genes involved in the maintenance of DNA integrity, including *BRCA1*, *BRCA2*, and *PALB2*. Importantly, four out of five patients with unstable genomes and/or a high *BRCA* mutational signature responded to platinum therapy, while none of three patients without these characteristics responded. This finding strongly suggests that whole-genome sequencing approaches may be used to define specific subgroups of patients and to tailor therapy accordingly [21].

Finally, the most comprehensive analysis to date (456 pancreatic cancer specimens) involving a combination of whole-genome and deep-exome sequencing, with gene copy number analysis, identified 32 persistently mutated genes and molecular pathways grouped into 10 signaling pathways and biological processes: *KRAS, TGF-β, WNT, NOTCH, ROBO/SLIT* signaling, *G1/S* transition, *SWI-SNF*, chromatin modification, DNA repair, and RNA processing. Additionally, RNA expression profiles of 232 pancreatic cancers defined four subtypes of pancreatic tumors: [1] squamous, [2] pancreatic progenitor, [3] immunogenic, and [4] aberrantly differentiated endocrine exocrine. The subtypes are associated with distinct genetic and epigenetic alterations, histopathological features, and survival rates [22].

Altogether, these studies have highlighted the complex genomic landscape of pancreatic cancer. Therefore, determining the role of the mutated genes in PDA initiation, progression, and maintenance, as well as the mechanisms of chemoresistance, is of paramount importance to uncover new therapeutic approaches. In this arduous task, mouse models of pancreatic cancer are playing a leading role and are helping to complement the genomic, transcriptomic, proteomic, and biological approaches employed to analyze human pancreatic cancer specimens.

Genetically Engineered Mouse Models as a Tool to Study Pancreatic Cancer Pathogenesis

The laboratory mouse, *Mus musculus*, owns characteristics that make it an ideal model system for cancer research including a small size, rapid reproduction, a relatively short lifespan of 3 years, and feasibility to recapitulate well the physiological and molecular features of human cancer. Additionally, its genome has been entirely sequenced, which has facilitated the extensive manipulation of the genome to generate genetically engineered mouse models (GEMMs) that express genetic alterations found in the human disease. GEMMs permit the ectopic expression of oncogenes (transgenic model), ablation of endogenous tumor suppressor genes (*knock-out* model), and physiological expression of oncogenes and negative dominant isoforms of tumor suppressor genes (*knock-in* model), in a spatiotemporal manner during tumor evolution to assess the role that specific genes and molecular pathways play during pancreatic cancer pathogenesis [23]. In the next sections of this chapter, the most relevant approaches to generate GEMMs of pancreatic cancer will be uncovered.

Ectopic Mouse Models of Pancreatic Cancer. Tumor Initiation and Cell of Origin

Early Transgenic Mouse Models

Pioneering approaches to generate mouse models of pancreatic cancer date back to the 1980s. Ornitz et al. [24] generated three transgenic lines that expressed the transforming *SV40 T-antigen* cDNA under the control of the rat *Elastase I* (*Ela-1*) promoter and enhancer, which drives the expression of exogenous cDNAs to the

pancreatic acinar cells from embryonic day E14. Newborn *Ela-1-SV40 T-antigen* transgenic mice showed hyperplastic pancreas at 2 weeks of age, and numerous pancreatic nodules at 10 weeks that rapidly progressed to exocrine pancreatic tumors, with mice from the three lines showing a median survival of 12.6–18.5 months [24].

A similar approach was used by Quaife et al. [25] to assess the oncogenic potential of $HRAS^{G12V}$ and *c-Myc*. *Ela-1-*$HRAS^{G12V}$ mice showed a dramatic development of pancreatic neoplasias including a massive acinar hyperplasia at embryonic day E14 that progressed to pancreatic dysplasia at E16 and acinar pancreatic tumor at E20. They monitored a cohort of 19 transgenic mice, finding that 14 out of 19 animals developed pancreatic cancer as newborns, with the remaining five mice developing pancreatic neoplasias as adults [25]. In contrast, none of the *Ela-1-c-Myc* mice developed pancreatic abnormalities [25]. Subsequently, Sandgren et al. [26] generated a slightly different *Ela-1-c-Myc* strain, in which the 3' noncoding region of the *c-Myc* gene, which is associated with mRNA instability [27], was replaced by the 3' noncoding region from the human *Growth Hormone* gene (*hGH*) that encodes a more stable mRNA. Interestingly, this strain developed mixed acinar/ductal pancreatic adenocarcinomas between 2 and 7 months of age [26]. The lack of oncogenicity of the *Ela-1-c-Myc* strain generated by Quaife et al. [25] was attributed to the low levels of expression of c-Myc. Finally, two groups showed that transgenic mice expressing the *Ela-1-TGFα* transgene developed hyperplasia, fibrosis, and pancreatic metaplasia with ductal-like features, with malignant transformation arising in mice older than 180 days at a low penetrance (<10%). These tumors, histologically classified as mixed cystic-papillary pancreatic tumors, originated from dysplastic tubular complexes. Furthermore, the tumors displayed an increased *EGFR* expression, strongly suggesting a robust influence of the TGFα/EGFR signaling pathway in pancreatic cancer development [28, 29].

Altogether, these early mouse models confirmed that pancreatic cancer pathogenesis is highly influenced by the oncogenic pathways activated. Thus, while expression in the pancreatic exocrine compartment of *SV40 T-antigen* and $HRAS^{G12V}$ induced acinar neoplasias, *c-Myc* and *TGFα* expression in acinar cells led to the development of pancreatic neoplasias with ductal features, strongly suggesting that pancreatic cancer could have an acinar origin. However, none of these mouse models described above were able to recapitulate the PanIN to PDA evolution observed in the human disease.

Kras Transgenic Mouse Models

Since oncogenic mutations in *KRAS* are found in over 90% of PDAs [6], and they are considered to be the earliest genetic event in pancreatic cancer development [3, 4], transgenic approaches were developed to generate mice expressing oncogenic *KRAS* in the pancreatic compartment with the hope of generating a mouse model that truly recapitulated the main features of PDA evolution. Grippo et al. [30] generated 10 transgenic lines carrying a transgene that expressed the human $KRAS^{G12D}$

cDNA under the control of the *Ela-1* promoter (*Ela-1-KRAS*^{G12D}). They found that mice from eight of those lines were smaller than control littermates at birth and displayed distended abdomens. Pancreata were found to be nodular or polycystic, and displayed a wide stromal reaction adjacent to a dysplastic epithelium with a glandular or papillary organization, and absence of normal ducts. Remarkably, mice from the remaining two transgenic lines showed a normal phenotype at birth and exhibited a nearly normal pancreatic histology, which was assumed to be the consequence of a reduced transgene expression and/or the site of transgene integration. As these mice aged, pancreata developed multifocal acinar hyperplasia, associated with focal dysplasia, fibrosis, and lymphocytic infiltration at 1–2 months of age. Older mice (6–18 months of age) developed acinar to ductal metaplasia (ADM) lesions, which are considered to be the precursor lesions of PDA [31]. However, none of the mice developed advanced pancreatic cancer, supporting the concept that genetic alterations were required for the progression of preinvasive lesions to frank malignancy. Importantly, this study indicated that the targeted activation of KRAS in the pancreatic acinar compartment is able to initiate pancreatic neoplasias with ductal features by inducing transdifferentiation of acinar cells.

In an effort to elucidate the cell of origin for PDA, Brembeck et al. [32] generated a transgenic strain that expressed the *KRAS*^{G12V} oncogene under the control of the *Cytokeratin-19* (*K19*) promoter, which is active in pancreatic ductal cells but not in other cell types of the pancreas. Importantly, *K19-KRAS*^{G12V} mice showed increased RAS activity in whole pancreatic extracts with lymphocytic infiltration observed around pancreatic ducts. However, expression of oncogenic *KRAS* in pancreatic ductal cells failed to initiate pancreatic neoplasias, introducing controversy about whether PanINs, in spite of the ductal-like properties, arise from mature pancreatic ductal cells.

Mist-Kras$^{G12D/+}$ *Knock-In* Mouse Model

A subsequent study by Tuveson et al. [33] shed light on the origin of the cancer-initiating cell in PDA. They generated a *knock-in* strain by cloning a *Kras*^{G12D} cDNA into the *Mist1* locus. Mist1 is a transcription factor that is expressed during pancreatic development after embryonic day E10.5 and required for correct pancreatic acinar organization. Pancreata from *Mist1-Kras*$^{G12D/+}$ mice displayed acinar and ductal metaplasia, and dysplasia. Acinar adenomas of solid or cystic nature were observed at 2 months of age, with invasive and metastatic pancreatic cancer developed after 3 months of age. Histologically, tumors were predominantly classified as cystic papillary neoplasms with acinar differentiation, with several specimens of mixed carcinomas with acinar and ductal features. However, glandular ductal adenocarcinomas were uncommon in this model. Interestingly, tumors recurrently developed the typical desmoplastic reaction present in human PDA including a rich collagenous stroma with accompanying fibroblasts. Moreover, the inactivation of the tumor suppressor gene *Trp53* cooperated with *Kras*^{G12D} to accelerate tumorigenesis (median survival of 6.5 months of the *Mist1-Kras*$^{G12D/+}$; *Trp53*$^{+/-}$ cohort

versus 10.8 months of the *Mist1-Kras*$^{G12D/+}$ cohort), confirming that PDA progression is supported by an accumulation of genomic alterations involving the activation of oncogenes and inactivation of tumor suppressor genes [3, 4]. This study strongly suggested that a pancreatic progenitor cell Mist1 positive represents a potential pancreatic cancer-initiating cell.

Overall, the ectopic mouse models described above have complemented histopathological analysis performed in human PDA specimens, providing vital information for understanding pancreatic cancer initiation. They have confirmed that oncogenic KRAS is able to initiate pancreatic neoplasias and have given insights into the cell of origin of PDA. However, a major limitation of these models is that none of them are able to recapitulate the main features of human PDA progression, with initial formation of PanIN lesions that progress to invasive and metastatic PDA. These drawbacks have been bypassed with the generation of more refined mouse models.

Conditional Kras Models of PanIN to PDA Progression. Cell of Origin

The remarkable development of gene targeting methods in embryonic stem cells during the last two decades have facilitated the physiological expression of oncogenes and inactivation of tumor suppressor genes in a spatiotemporal manner, leading to the generation of more sophisticated compound mutant mice that have closely recapitulated the features of human PDA. The generation of GEMMs harboring different sets of mutations have reinforced the genetic basis of the pancreatic cancer progression model defined by Hruban et al. [3, 4], allowing the study of diverse aspects of the genetics and biology of PDA.

The first GEMM that faithfully resembled the human disease was generated in 2003 by David Tuveson's laboratory [34]. Tuveson's laboratory used the *LSL-Kras*$^{G12D/+}$ *knock-in* mouse strain [35] to generate compound mutant mice that conditionally expressed oncogenic *Kras*^{G12D} in pancreatic progenitor cells. The *LSL-Kras*$^{G12D/+}$ strain harbors an endogenous *Kras*^{G12D} mutant allele transcriptionally silenced by a STOP cassette flanked by LoxP sites (LoxP-Stop-LoxP, LSL) cloned upstream of the targeted *Kras*^{G12D} Exon1. After *Cre* recombinase expression, the LSL cassette is removed thereby allowing the expression of oncogenic *Kras*^{G12D} in a spatiotemporal manner (Fig. 2). *LSL-Kras*$^{G12D/+}$ mice were interbred with two mouse models that expressed *Cre* recombinase during the embryonic development leading to the expression of *Kras*^{G12D} in all pancreatic lineages: the *Pdx1-Cre* transgenic strain (which expresses *Cre* in the prepancreatic endoderm from E8**.**5) and the *Ptf1a/P48-Cre knock-in* strain (which expresses *Cre* in the prepancreatic endoderm from E9.5). Compound mutant mice *LSL-Kras*$^{G12D/+}$*; Pdx1-Cre* and *LSL-Kras*$^{G12D/+}$; *Ptf1a/P48-Cre* (known as *KC*) developed preinvasive neoplasias, catalogued as PanIN lesions, with complete penetrance [34]. Remarkably, *KC* mice recapitulated the full spectrum of lesions seen in the human disease, starting with the development of PanIN-1 lesions in young mice that progressed through

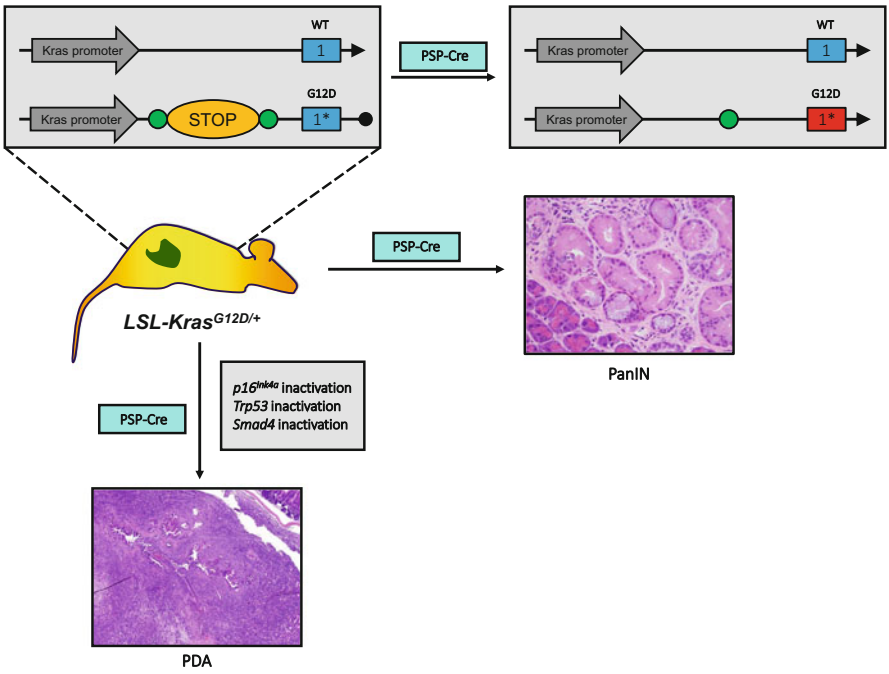

Fig. 2 Conditional GEMMs of pancreatic cancer. The *LSL-Kras*$^{G12D/+}$ *knock-in* strain carries a *Kras*^{G12D} allele transcriptionally silenced by a STOP cassette flanked by LoxP sites (*green circles*). *Cre* recombinase expression driven by pancreas specific promoters (PSP) removes the LoxP-STOP-LoxP cassette, thereby allowing *Kras*^{G12D} expression in the pancreas compartment, which in turn induces the development of PanINs. PanINs can progress to frank malignancy after inactivation of tumor suppressor genes, including *p16*^{Ink4a}, *Trp53*, or *Smad4*

PanIN-2 and PanIN-3 in older mice, with a subset of mice developing frank PDA usually not before 12 months of age [34]. Further cellular and molecular characterization revealed that murine PanIN, as human PanIN, expressed high levels of mucins and the epithelial ductal cell marker Cytokeratin-19 [34], supporting the idea that PDA derived from normal ductal cells or their precursors. However, and as mentioned above, the transgenic expression of the *Kras*^{G12V} oncogene from the *K19* promoter failed to induce PanIN or PDA [32], introducing controversy about whether PanIN, despite presenting ductal-like properties, arise from a mature ductal cell or from a progenitor cell of any lineage.

A few years later, Carriere et al. [36] gave additional insights into the cellular origin of PanIN. They generated *LSL-Kras*$^{G12D/+}$*; Nestin-Cre* mice, where *Cre* recombinase expression was directed to a population of pancreatic exocrine progenitors that express *Nestin* during embryonic days E10.5–12.5. They found that young *LSL-Kras*$^{G12D/+}$*; Nestin-Cre* mice (<6 months) developed PanIN-1 lesions at a similar frequency as observed in *LSL-Kras*$^{G12D/+}$*; Pdx1-Cre* mice, which expressed *Kras*^{G12D} in all pancreatic lineages. None of the *LSL-Kras*$^{G12D/+}$*;*

Nestin-Cre mice developed high-grade PanIN or PDA lesions due to the Nestin-mediated $Kras^{G12D}$ expression in the central nervous system that led to dramatic lethality after 6 months due to neurological problems. This study has supported that an exocrine progenitor lineage, rather than mature pancreatic ductal or acinar cells, may be the cell origin of pancreatic cancer.

Importantly, a different PanIN to PDA progression mouse model generated by Mariano Barbacid's laboratory [37] supported a nonductal origin of PDA. This model was based on the use of the *LSL-*$Kras^{G12Vgeo/+}$ mouse strain [38], which harbors a conditional $Kras^{G12V}$ and *β-geo* bicistronic allele transcriptionally silenced by a LSL cassette. *LSL-*$Kras^{G12Vgeo/+}$ mice were interbred with *Elastase-tTA* and *tetO-Cre* mice to generate the compound mutant strain *LSL-*$Kras^{G12Vgeo/+}$*; Elastase-tTA; tetO-Cre* [37]. The *Elastase-tTA* strain expresses the tTA transactivator in the pancreatic acinar/centroacinar compartment, thereby allowing the expression of *Cre* recombinase from the *tetO-Cre* allele when doxycycline was not supplemented in the drinking water. This model showed that *Cre*-mediated $Kras^{G12V}$ expression in pancreatic acinar/centroacinar cells of embryos or newborns, but not adult mice, faithfully mimicked the development of ADM lesions, PanIN lesions, and invasive PDA observed in the *LSL-*$Kras^{G12D/+}$*; Pdx1-Cre* and *LSL-*$Kras^{G12D/+}$; *Ptf1a/P48-Cre* models, which expressed the $Kras^{G12D}$ oncogene in all pancreatic lineages [34]. Interestingly, PanIN to PDA progression was dramatically accelerated after caerulein-induced chronic pancreatitis, confirming that inflammation synergizes with oncogenic Kras to promote pancreatic cancer. Overall, this study strongly suggests that PDA initiates by differentiation of acinar/centroacinar cells, or their precursors, into ductal-like cells [37].

Collectively, PanIN to PDA progression mouse models have supported a non-ductal origin of pancreatic cancer. Instead, PanINs seem to be originated either from pancreatic progenitor cells [34] or transdifferentiating acinar cell [31, 37, 39–41], which would support observations proposing that ADM might be the earliest pancreatic lesion and, as such, the precursor of PanIN-1 lesions [31, 39, 40].

Accelerated Mouse Models of PDA Progression. Confirmation of the Genetic Progression Model

GEMMs expressing endogenous levels of Kras oncogenes in the pancreatic compartment have supported the PDA progression model proposed by Hruban et al. [3, 4], which postulates that oncogenic mutations in the *KRAS* gene are the initiating genetic event in PDA development and induce low-grade PanIN lesions that progress to PDA following the acquisition of additional epigenetic and genetic alterations (Fig. 1). The genetic basis of this progression model has been further validated by the generation of GEMMs that combined the activation of Kras oncogenes with inactivation of known tumor suppressor genes in the pancreatic compartment (Fig. 2). In the framework of this chapter, the most relevant GEMMs of pancreatic cancer generated during the last decade will be discussed (Table 1).

Table 1 Endogenous GEMMs of pancreatic cancer

GEMM	Pancreatic phenotype	Metastasis	Survival (S)/tumor latency (L)	Comments	References
LSL-Kras$^{G12D/+}$; Pdx1-Cre	PanIN, PDA	>50%	>12 months (S)	Slow PanIN to PDA progression	[34]
LSL-Kras$^{G12D/+}$; Ptf1a/P48-Cre	PanIN, PDA	>50%	>12 months (S)	Slow PanIN to PDA progression	[34]
LSL-Kras$^{G12D/+}$; Nestin-Cre	PanIN	No	~6 months (S)	Lethality due to Cre expression in brain	[36]
LSL-Kras$^{G12Vgeo/+}$; Elastase-tTA; tetO-Cre	PanIN, PDA	No	>12 months (S)	Slow PanIN to PDA progression	[37]
LSL-Kras$^{G12D/+}$; Ink4a/Arf$^{flox/flox}$; Pdx1-Cre	PanIN, PDA	11%	8.5 weeks (L)	Micrometastasis only	[47, 48]
LSL-Kras$^{G12D/+}$; Ink4a/Arf$^{flox/+}$; Pdx1-Cre	PanIN, PDA	69%	34.2 weeks (L)		[47]
LSL-Kras$^{G12D/+}$; Trp53$^{flox/flox}$; Pdx1-Cre	PanIN, PDA	No	6.2 weeks (L)		[48]
LSL-Kras$^{G12D/+}$; Trp53$^{flox/+}$; Pdx1-Cre	PanIN, PDA	33%	21.8 weeks (L)		[48]
LSL-Kras$^{G12D/+}$; Trp53$^{flox/+}$; p16Ink4a$^{+/-}$; Pdx1-Cre	PanIN, PDA	25%	14.7 weeks (L)		[48]
LSL-Kras$^{G12D/+}$; Trp53$^{flox/+}$; p16Ink4a$^{-/-}$; Pdx1-Cre	PanIN, PDA	25%	13.1 weeks (L)		[48]
LSL-Kras$^{G12D/+}$; LSL-Trp53$^{R172H/+}$; Pdx1-Cre	PanIN, PDA	63%	5 months (S)		[51]
LSL-Kras$^{G12D/+}$; Tgfbr2$^{flox/+}$; Ptf1a/P48-Cre	PanIN, PDA	50%	33.6 weeks (S)		[56]
LSL-Kras$^{G12D/+}$; Tgfbr2$^{flox/flox}$; Ptf1a/P48-Cre	PanIN, PDA	12%	59 days (S)	Only long survivors develop metastasis	[56]
LSL-Kras$^{G12D/+}$; Smad4$^{flox/flox}$; Pdx1-Cre	PanIN, IPMN	No	13.1 weeks (L)		[57]

(*continued*)

Table 1 (continued)

GEMM	Pancreatic phenotype	Metastasis	Survival (S)/tumor latency (L)	Comments	References
LSL-Kras$^{G12D/+}$; Smad4$^{flox/flox}$; Ptf1a/P48-Cre	PanIN, IPMN, PDA	No	15.7 weeks (L)	IPMN to PDA progression	[57]
LSL-Kras$^{G12D/+}$; Smad4$^{flox/flox}$; Ink4a/Arf$^{flox/+}$; Ptf1a/P48-Cre	PanIN, IPMN, PDA	37.5%	14 weeks (L)	IPMN to PDA progression	[57]
LSL-Kras$^{G12D/+}$; Smad4$^{flox/flox}$; Ink4a/Arf$^{flox/+}$; Pdx1-Cre	PanIN, IPMN, PDA		12.6 weeks (L)	IPMN to PDA progression	[57]
LSL-Kras$^{G12D/+}$; Smad4$^{flox/+}$; Pdx1-Cre	PanIN, cystic lesion	No	8 months (S)	Lethality due to gastric carcinomas	[60]
LSL-Kras$^{G12D/+}$; Smad4$^{flox/+}$; Ptf1a/P48-Cre	PanIN, MCN, PDA	41%	15 months (S)	MCN to PDA progression	[60]
LSL-Kras$^{G12D/+}$; Smad4$^{flox/flox}$; Ptf1a/P48-Cre	PanIN, MCN, PDA	18%	8 months (S)	Accelerated MCN to PDA progression	[60]
LSL-Kras$^{G12D/+}$; Elastase-TGFa; Ptf1a/P48-Cre	PanIN, IPMN, PDA	50%	7 months (S)	IPMN to PDA progression	[62]
LSL-Kras$^{G12D/+}$; Tif1γ$^{flox/flox}$; Pdx1-Cre	IPMN	No	ND		[63]

PDA Pancreatic Ductal Adenocarcinoma, *PanIN* Pancreatic Intraepithelial Neoplasia, *IPMN* Intraductal Papillary Mucinous Neoplasia *MCN* Mucinous Cystic Neoplasia, *ND* Not determined

Information of other mouse models that have helped to delineate the pathogenesis of PDA can be found in recent reviews [11, 42, 43].

Oncogenic Kras and *Ink4a/Arf* Inactivation

The *INK4A/ARF* (*CDKN2A*) locus encodes for the tumor suppressors $p16^{INK4A}$ and $p14^{ARF}$ ($p19^{ARF}$ in mice) [44, 45]. Genetic or epigenetic inactivation of the cyclin-dependent kinase inhibitor $p16^{INK4A}$ is a relatively early event in PDA development, and occurs in over 95% of human PDA samples (Fig. 1). Additionally, homozygous deletions of the *INK4A/ARF* locus, affecting both $p16^{INK4A}$ and the TP53 activator $p14^{ARF}$, happen in 40% of pancreatic cancers [46]. Early efforts to delineate the role of the *INK4A/ARF* locus during PDA development were performed by Ronald Depinho's laboratory. In an initial study, they interbred the *LSL-Kras$^{G12D/+}$* strain

with conditional *knock-out* mice harboring a $p16^{Ink4a}/p19^{Arf}$ allele with exons 2–3 flanked by LoxP sites. They generated *LSL-*$Kras^{G12D/+}$*; Ink4a/*$Arf^{flox/flox}$; *Pdx1-Cre* mice and found that the biallelic ablation of $p16^{Ink4a}/p19^{Arf}$ driven by the *Pdx1-Cre* allele cooperated with $kras^{G12D}$ to accelerate PanIN to PDA progression. Mice developed PanIN-1 lesions as early as 3 weeks of age that rapidly progressed through PanIN-2/3 lesions to a highly aggressive locally invasive PDA associated with the formation of micrometastasis in 11% of the mice [47, 48]. In a subsequent study, they generated *LSL-*$Kras^{G12D/+}$*; Ink4a/*$Arf^{flox/+}$; *Pdx1-Cre* mice and showed that monoallelic ablation of the $p16^{Ink4a}/p19^{Arf}$ locus and concomitant expression of $Kras^{G12D}$ led to the development of PDA with a longer latency compared with *LSL-*$Kras^{G12D/+}$*; Ink4a/*$Arf^{flox/flox}$; *Pdx1-Cre* mice (34.2 vs. 8.5 weeks, respectively). Additionally, $Kras^{G12D}$*; Ink4a/Arf* heterozygous PDAs showed enhanced metastatic potential compared with $Kras^{G12D}$*; Ink4a/Arf* null PDAs (69% vs. 11% mice showing metastasis, respectively) [47]. This invasive phenotype was associated with longer survival of the heterozygous *Ink4a/Arf* mice.

Oncogenic Kras and *Trp53* Inactivation

The tumor suppressor TP53 is considered the main guardian of the genome, and it is implicated in pivotal biological processes, including cell cycle arrest, DNA repair, and apoptosis [49]. Inactivation of the transcription factor TP53 (encoded by the human gene *TP53*) is a common genetic event in PDA, and it is strongly associated with PDA progression. Missense mutations in *TP53* are frequently associated with loss of the second wild-type allele, and they are found in 70–76% human PDA samples [13, 50] (Fig. 1).

Mutations in the *TP53* gene are distributed along the coding sequence with a strong prevalence in exons 4–9, which encode the DNA-binding domain of the protein. The three main mutation hotspots in the *TP53* gene found in human PDA are within this DNA-binding domain, and affect the residues R175, R248, and R273 (http://p53.free.fr). Over a decade ago, Hingorani et al. [51] investigated the impact of mutations in the mouse Trp53 DNA-binding domain in PDA development. They engineered a *knock-in* mouse strain harboring a conditional mouse ortholog of the human $TP53^{R175H}$ gene, *LSL-*$Trp53^{R172H/+}$. They generated *LSL-*$Kras^{G12D/+}$*;* *LSL-*$Trp53^{R172H/+}$*; Pdx1-Cre* (*KPC*) mice and found that the physiological expression in the pancreatic compartment of $Trp53^{R172H}$ in the context of $Kras^{G12D}$ led to the development of early PanIN lesion at 4 weeks of age that progressed to invasive PDA with mice showing a median survival of 5 months. Furthermore, 63% of *KPC* mice developed metastasis, with 59% of mice developing ascites [51]. Remarkably, PDA cell lines established from KPC tumors displayed aneuploidy and chromosomal instability, mirroring one of the main hallmarks of human PDA and strongly supporting the pivotal role of TP53 inactivation in PDA development.

The implication of the Trp53 inactivation during the pathogenesis of PDA was further decoded by combining the expression of $Kras^{G12D}$ with the heterozygous (*LSL-*$Kras^{G12D/+}$*; Trp53*$^{flox/+}$*; Pdx1-Cre*, $KP^{f/+}C$) or homozygous (*LSL-*$Kras^{G12D/+}$*;*

Trp53$^{flox/flox}$*; Pdx1-Cre*, *KP*$^{f/f}$*C*) deletion of *Trp53*. While *LSL-Kras*$^{G12D/+}$*; Pdx1-Cre* mice showed a tumor latency of 57 months, both heterozygous and homozygous inactivation of *Trp53* significantly accelerated PDA development with tumor latencies of 21.8 and 6.2 months, respectively [47]. Interestingly, and in contrast to the expression of mutant *Trp53*^{R172H} [51], Trp53 null PDAs displayed low metastatic potential. Indeed, only 33% of *KP*$^{f/+}$*C* mice developed metastasis, while none of the *KP*$^{f/f}$*C* showed metastatic deposits [47]. This behavior was confirmed in an independent study by Morton et al. [52]. They generated and monitored *KP*$^{f/+}$*C* and *KPC* compound mutant mice. While both strains showed a similar median survival (113 vs. 123 days, respectively), the incidence of metastasis was significantly higher in PDAs expressing *Trp53*^{R172H} (65%) compared with Trp53 null PDA (0%) [52]. Interestingly, Weissmueller et al. [53] have recently reported that mutant Trp53-mediated platelet-derived growth factor receptor beta (PDGFRβ) induction plays a critical role in the metastatic behavior of PDA harboring *Trp53* missense mutations. The authors showed that the inhibition of the p73/NF-Y complex by mutant Trp53 leads to the upregulation of PDGFRβ, which is strongly correlated with metastatic potential of PDA cells, unveiling one mechanism by which the gain-of-function activity of mutant Trp53 promotes invasion and metastasis in PDA [53].

Rozenblum et al. [50] showed that a significant number of PDAs, 25 out of 38, harbor inactivating mutations of both *p16*^{INK4A} and *TP53*, suggesting that the ablation of both tumor suppressors could be cooperating events during PDA progression. Bardeesy et al. [47] investigated the functional interaction of the dual *Trp53* and *p16*^{Ink4a} inactivation in PDA development. They interbred *LSL-Kras*$^{G12D/+}$*; Pdx1-Cre* mice with mice harboring homozygous or heterozygous *Trp53*^{flox} and/or *p16*$^{Ink4a\text{-}KO}$ alleles, and found that the inactivation, either alone or in combination, of *Trp53* and/or *p16*^{Ink4a} cooperated with *Kras*^{G12D} to accelerate PDA development. Additionally, they found that in the context of *Kras*^{G12D} and *Trp53* heterozygosity, both the heterozygous and homozygous deletion of *p16*^{Ink4a} dramatically shortened tumor latency (21.8 months (p16^{Ink4a} wild-type) versus 14.7 months (p16$^{Ink4a\text{-}KO}$ heterozygous) and 13.1 months (p16$^{Ink4a\text{-}KO}$ homozygous)), suggesting that inactivating mutations in *Trp53* and *p16*^{Ink4a} cooperate during PDA development. Furthermore, while in a *Kras*^{G12D} and Trp53 null background, inactivation of *p16*^{Ink4a} does not impact significantly tumor latency; the heterozygous or homozygous deletion of *Trp53* drastically reduces tumor latency in a *Kras*^{G12D} and *p16*^{Ink4a} null background, strongly suggesting that Trp53 functions as a more powerful barrier to PDA development [47].

Oncogenic Kras and TGFβ Signaling Inactivation. Role of the TGFβ Pathway in the Development of Cystic Neoplasias

The fourth most common genetic event involved in human PDA development is the inactivation of members of the Transforming Growth Factor beta (TGFβ) pathway, including *DPC4/SMAD4* (found mutated in over 50% of PDA specimens), *TGFβRI* (<5%), and *TGFβRII* (<5%) [14, 15]. Inactivation of the TGFβ pathway is believed

to occur in high-grade PanIN lesions [5] (Fig. 1), and it is associated with a highly invasive phenotype [54]. The TGFβ signaling pathway is involved in many cellular processes including cell growth, cell differentiation, and apoptosis. In normal and premalignant cells, TGFβ signaling preserves cellular homeostasis and exerts a tumor suppressor role. However, neoplastic cells are able to evade the TGFβ tumor-suppressive properties, instead using TGFβ to enhance transformation, invasion, and tumor dissemination [55].

GEMMs have been employed to understand the role of the TGFβ signaling pathway in the pathogenesis of PDA. Ijichi et al. [56] conditionally inactivated *Tgfbr2* in pancreatic progenitor cells by interbreeding mice carrying a *Tgfbr2* conditional *knock-out* allele, which harbored an exon 2 flanked by LoxP sites, with *Ptf1a/P48-Cre* mice. *Tgfbr2$^{flox/flox}$; Ptf1a/P48-Cre* mice developed normally and did not show any pancreatic abnormality, suggesting that Tgfbr2 does not play a critical role in pancreatic homeostasis. When the ablation of *Tgfbr2* was combined with the expression of *Kras^{G12D}*, mice showed a dramatic acceleration of PDA development. *LSL-Kras$^{G12D/+}$; Tgfbr2$^{flox/flox}$; Ptf1a/P48-Cre* mice exhibited abdominal distension associated with ascites and pancreatic tumors at ~6–7 weeks of age, and a median survival of 59 days. Most of the mice from this cohort were sacrificed at 7–10 weeks of age and did not show any distant metastasis. However, three mice that survived up to 24–27 weeks developed distant metastases in the liver, lungs, and diaphragm, as well as duodenal invasion and peritoneal dissemination. These findings confirmed the highly metastatic potential conferred by the inactivation of the TGFβ signaling pathway, and suggested that the low number of metastases detected in *LSL-Kras$^{G12D/+}$; Tgfbr2$^{flox/flox}$; Ptf1a/P48-Cre* mice may be due to their early lethality. To verify this hypothesis, the authors generated a cohort that combined *Kras^{G12D}* expression with the heterozygous inactivation of *Tgfbr2*. Interestingly, *LSL-Kras$^{G12D/+}$; Tgfbr2$^{flox/+}$; Ptf1a/P48-Cre* mice showed a median survival of 33.6 weeks and, importantly, 50% of the mice developed distant metastases mainly in the liver and lungs. Remarkably, heterozygous *Tgfbr2* mice retained the *Tgfbr2* wild-type allele confirming that *Tgfbr2* haploinsufficiency, in the context of oncogenic *Kras* expression, leads to PDA progression [56].

Additional insights into the significance of the TGFβ pathway in pancreatic cancer emerged from a study performed by Bardeesy et al. [57]. They generated a *Smad4* conditional *knock-out* allele, which harbored exons 8–9 flanked by LoxP sites. The analysis of *Smad4$^{flox/flox}$; Pdx1-Cre* and *Smad4$^{flox/flox}$; Ptf1a/p48-Cre* compound mutant mice revealed that the inactivation of *Smad4* did not impact in the normal development of the pancreas, supporting previous findings showing that *Tgfbr2* disruption did not affect pancreatic development [56]. When *Smad4* ablation was concomitant with *Kras^{G12D}* activation in pancreatic progenitor cells, *LSL-Kras$^{G12D/+}$; Smad4$^{flox/flox}$; Pdx1-Cre* and *LSL-Kras$^{G12D/+}$; Smad4$^{flox/flox}$; Ptf1a/P48-Cre* mice showed rapid tumor progression, with an increase in the number and size of low-grade PanIN lesions, at 4 weeks of age, compared with mice expressing *Smad4*. Pancreatic lesions rapidly progressed to extensive IPMN and advanced PanIN lesions by 8 weeks of age. Overall, *LSL-Kras$^{G12D/+}$; Smad4$^{flox/flox}$; Ptf1a/P48-Cre* mice showed a median survival of 15.7 weeks, with 100% of the

mice (n = 12) exhibiting IPMN and two out of twelve also presenting PDA. Furthermore, *LSL-Kras$^{G12D/+}$; Smad4$^{flox/flox}$; Pdx1-Cre* mice showed a median survival of 13.1 weeks, with five out of eight mice developing IPMN, and five mice also presenting gastric cancer due to the expression of Pdx1 in the foregut. None of the *LSL-Kras$^{G12D/+}$; Smad4$^{flox/flox}$; Pdx1-Cre* mice displayed PDA. These findings supported the role of Smad4 as a barrier for the progression of Kras^{G12D}-initiated PanINs to pancreatic cancer. Furthermore, the development of IPMN indicates that Smad4 may have a critical role in the formation of pancreatic cystic neoplasias.

Further genetic studies incorporating an *Ink4a/Arf^{flox}* conditional allele [57] revealed that Smad4 deficiency altered the tumor spectrum associated with the combined expression of *Kras^{G12D}* and *Ink4a/Arf* heterozygosity (60% of mice presenting PDA (n = 10) and a tumor-free survival of 38 weeks) or *Ink4a/Arf* homozygosity (100% of mice presenting PDA (n = 6) and a tumor-free survival of 8.6 weeks). Accordingly, *Ink4a/Arf* heterozygosity cooperated with *Kras^{G12D}* and *Smad4* ablation to accelerate pancreatic tumor progression. Thus, *Kras$^{G12D/+}$; Smad4$^{flox/flox}$; Ink4a/Arf$^{flox/+}$*; *Ptf1a/P48-Cre* mice showed a tumor-free survival of 14 weeks associated with the development of IPMN (1 out of 13 mice), PDA (12 out of 13 mice), and both IPMN and PDA (4 out of 13 mice). Furthermore, *LSL-Kras$^{G12D/+}$; Smad4$^{flox/flox}$; Ink4a/Arf$^{flox/+}$*; *Pdx1-Cre* mice showed a tumor-free survival of 12.6 weeks, with mice presenting IPMN (4 out of 12), PDA (4 out of 12), and gastric cancer (8 out of 12). On the other hand, the homozygous inactivation of *Ink4a/Arf* did not significantly affect tumor-free survival. Thus, *LSL-Kras$^{G12D/+}$; Smad4$^{flox/flox}$; Ink4a/Arf$^{flox/flox}$*; *Ptf1a/P48-Cre* mice showed tumor-free survival of 8.8 weeks associated with the development of PDA (4 out of 4 mice). Furthermore, *LSL-Kras$^{G12D/+}$; Smad4$^{flox/flox}$; Ink4a/Arf$^{flox/flox}$*; *Pdx1-Cre* mice displayed a tumor-free survival of 7.4 weeks, with 3 out of 10 developing IPMN, while 9 out of 10 presented PDA. Again, a considerable number of these mice from the Pdx1 cohort (50%) developed gastric cancer due to the activity of the *Pdx1* promoter in the foregut [57] .

Collectively, this study showed that *Smad4* deficiency leads to the development of IPMN in a *Kras^{G12D}* context (17/20 mice), with a low proportion of mice (2/20) presenting frank PDA. Interestingly, the inactivation of the tumor suppressor *Ink4a/Arf* in a *Kras^{G12D}* and *Smad4* null background dramatically increased PDA incidence, with 64% of heterozygous and 92% of homozygous mice developing PDA. These findings clearly confirm that the genetic landscape strongly determines tumor evolution.

Strikingly, mutations in *SMAD4* are infrequent in human IPMN, and are instead more common in human MCN [58, 59]. Genetic approaches using GEMMs have supported the human studies, delineating the role of the TGFβ pathway in the pathogenesis of MCN. Izeradjene et al. [60] interbred a *Smad4* conditional *knock-out* strain harboring an exon 8 flanked by LoxP sites [61] with *LSL-Kras$^{G12D/+}$*; *Pdx1-Cre* mice. Pancreata from *LSL-Kras$^{G12D/+}$; Smad4$^{flox/+}$; Pdx1-Cre* mice revealed the development of PanINs and macroscopic cystic lesions, although the early lethality of this cohort due to the development of gastric carcinomas (median

survival of approximately 8 months) precluded the analysis of late stages of the disease. To overcome the lethality induced by the expression of Pdx1 in the gastric epithelium, they performed a subsequent study in a *Ptf1a/p48-Cre* background, which restrains the expression of *Cre* recombinase to the pancreas compartment. Mice with heterozygous *Smad4* inactivation combined with $Kras^{G12D}$ activation, $LSL\text{-}Kras^{G12D/+}$; $Smad4^{flox/+}$; *Ptf1a/P48-Cre*, showed similar median survival to $LSL\text{-}Kras^{G12D/+}$; *Ptf1a/P48-Cre* mice. Remarkably, pancreata examination of heterozygous *Smad4* mice revealed the development of palpable abdominal masses in the body and tail that corresponded with large mucinous cystic lesions classified as MCN. PanIN lesions were also observed, although they usually were of a lower grade compared with those found in age-matched *Smad4* wild-type control littermates. Mice with homozygous *Smad4* ablation, $LSL\text{-}Kras^{G12D/+}$; $Smad4^{flox/flox}$; *Ptf1a/P48-Cre*, showed reduced median survival compared with heterozygous counterparts (8 vs. 15 months, respectively) associated with accelerated development of MCN. Interestingly, $LSL\text{-}Kras^{G12D/+}$; $Smad4^{flox/+}$; *Ptf1a/P48-Cre* and $LSL\text{-}Kras^{G12D/+}$; $Smad4^{flox/flox}$; *Ptf1a/P48-Cre* mice showed reduced metastatic behavior compared to the KPC mice, which correlated with the less aggressive phenotype showed by the cystic pancreatic neoplasias compared with PDA in humans [61].

Other GEMMs have provided additional insights into the molecular basis of pancreatic cystic neoplasia development. Siveke et al. [62] interbred *Elastase-Tgfα* transgenic mice with $LSL\text{-}Kras^{G12D/+}$; *Ptf1a/P48-Cre* mice. They found that concomitant expression of *Tgfα* and $Kras^{G12D}$ led to the development of cystic papillary neoplasias with resemblance to IPMNs that rapidly progressed to invasive and metastatic PDA [62]. Moreover, Vincent et al. [63] investigated the role of the TGFβ signaling regulator Transcriptional Intermediary Factor 1 gamma (Tif1γ) in pancreatic cancer development. They generated $LSL\text{-}Kras^{G12D/+}$; $Tif1\gamma^{flox/flox}$; *Pdx1-Cre* mice and found that the deletion of *Tif1γ* in pancreatic progenitor cells cooperated with $Kras^{G12D}$ to induce pancreatic tumors reminiscent of human IPMNs [63].

Taken together, the mouse models described above have helped to understand the genetics and biology of pancreatic cancer, establishing that aberrations in the TGFα and TGFβ pathways play a critical role in the generation of both PanINs and cystic neoplasias, including IPMNs and MCNs [16], that eventually progress to invasive PDA.

Mouse Models to Study the Role of Oncogenic Kras in PDA Maintenance

Activating mutations in *KRAS* are required for PDA development. Given that PDA is detected virtually only once the tumor is established, understanding of the role of oncogenic KRAS in PDA maintenance is of utmost importance to unveil molecular pathways with therapeutic relevance to tackle this disease. During the last few years, a new generation of GEMMs have helped to delineate the role of oncogenic Kras in PDA progression and maintenance. The Pasca di Magliano laboratory [64]

engineered a *tetO-Kras*^{G12D} regulatable transgenic allele which allows the expression of *Kras*^{G12D} when doxycycline is supplemented in the diet. They interbred *tetO-Kras*^{G12D} mice with *Ptf1a/P48-Cre* and *Rosa26-LSL-rtTA-IRES-EGFP* mice to generate the compound mutant strain *tetO-Kras*^{G12D}; *Rosa26-LSL-rtTA-IRES-EGFP*; *Ptf1a/P48-Cre* (known as *iKras**). *iKras** mice express *Cre* recombinase in the pancreas compartment from E9.5 that, in turn, removes the LSL cassette of the *Rosa26-LSL-rtTA-IRES-EGFP* allele allowing the expression of both the *rtTA* transactivator and the *EGFP* reporter in pancreatic progenitor cells. Once doxycycline is administrated in the drinking water, rtTA is activated, leading to *Kras*^{G12D} expression from the *tetO-Kras*^{G12D} allele. Importantly, upon doxycycline withdrawal *Kras*^{G12D} expression is reversed [64] (Fig. 3).

iKras* models have provided a valuable tool for understanding the role of oncogenic Kras in PDA maintenance. When *Kras*^{G12D} was abrogated in PanIN lesions established in **iKras* mice, preinvasive lesions reverted both with and without induction of acute pancreatitis, confirming that oncogenic Kras is required for PanIN maintenance [64]. Furthermore, the authors used this system to investigate advanced PDA. Thus, the *iKras** strain was introduced in a *Trp53*$^{+/-}$ background. Interestingly, both PanINs and PDA lesions established in *iKras*-p53*$^{+/-}$ mice regressed after *Kras*^{G12D} inactivation. This regression was associated with MAPK downregulation, although unlike iKras* PanIN lesions, pancreata did not fully recover their normal histology, and areas of metaplasia surrounded by fibrosis remained in the pancreatic parenchyma after *Kras*^{G12D} ablation [64].

In a parallel study, Ying et al. [65] engineered a *tetO_Lox-Stop-Lox-Kras*^{G12D} transgenic strain that was interbred with *Rosa26-LSL-rtTA-IRES-GFP* and *Ptf1a/P48-Cre* mice to generate *tetO_Lox-Stop-Lox-Kras*^{G12D}; *Rosa26-LSL-rtTA-IRES-GFP*; *Ptf1a/P48-Cre* compound mutant mice (known as *iKras*). Significantly, tumor regression was observed after *Kras*^{G12D} inactivation in both the *iKras* and *iKras-Trp53*$^{+/-}$models, supporting previous findings using the *iKras** model [64]. Strikingly, the authors showed that oncogenic Kras promotes metabolic reprogramming in neoplastic cells to sustain tumorigenesis. Indeed, they found that *Kras*^{G12D} inactivation led to inhibition of glucose uptake with a decrease in glycolytic intermediates [65].

Overall, oncogenic Kras regulatable mouse models have established that pancreatic cancer is strictly dependent on *Kras*^{G12D} expression, providing an unbiased genetic model to understand the molecular mechanisms involved in PDA progression and maintenance with the aim of identifying molecular targets for therapeutic interventions.

Mouse Models to Identify and Validate Human Pancreatic Cancer Genes

Pancreatic cancer evolves as a consequence of genetic alterations acquired during the progression of the disease. Indeed, whole-genome sequencing approaches performed in human pancreatic cancer specimens have exposed the complexity and heterogeneity of the tumor genomes, unveiling a significant number of novel candidates involved in the pathogenesis of pancreatic cancer [17, 18, 21, 22]. This

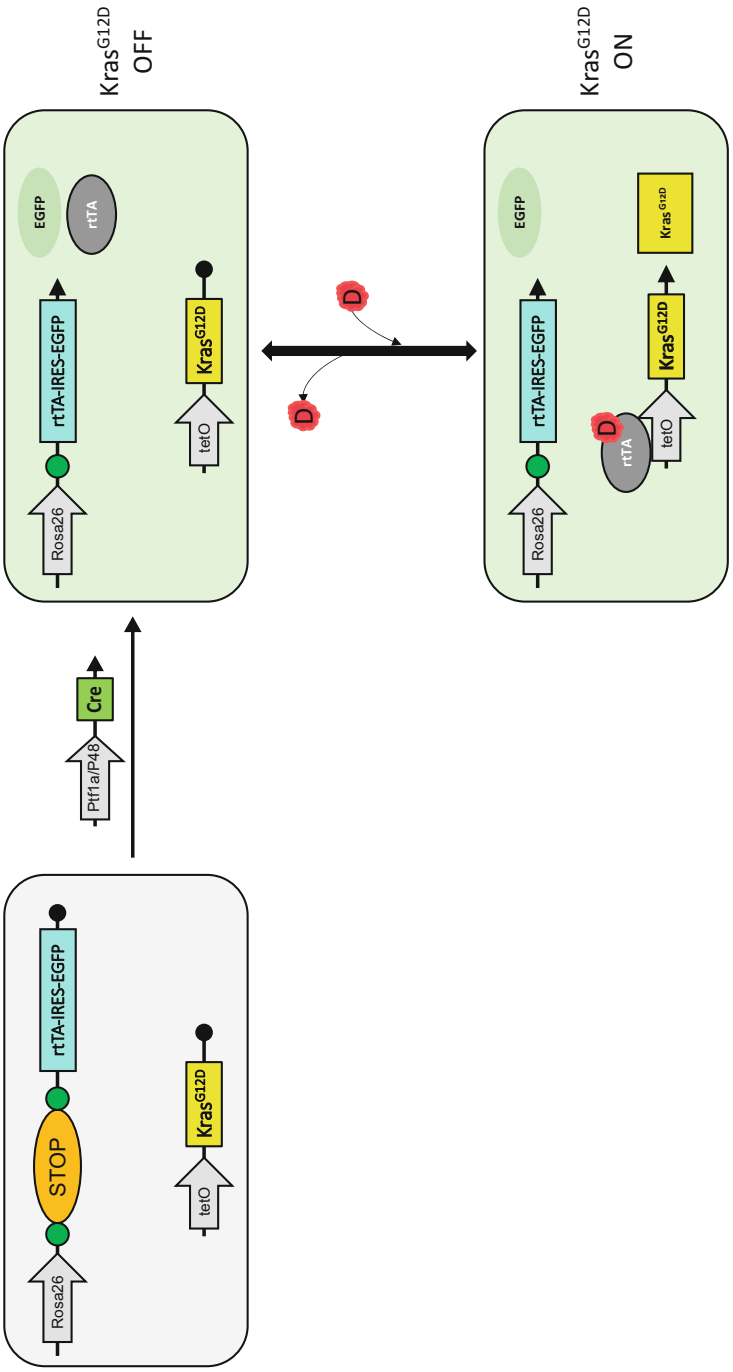

Fig. 3 ***iKras* mouse model.** *Ptf1a/P48*-driven *Cre* expression removes the LoxP-STOP-LoxP cassette from the *Rosa26-LSL-rtTA-IRES-EGFP* allele, thereby allowing the expression of the *rtTA* transactivator and the green fluorescent protein (*EGFP*). When doxycycline (D) is administrated, rtTA binds to the tetracycline operator (tetO) inducing the expression of $Kras^{G12D}$. When doxycycline is removed, rtTA is inactivated and $Kras^{G12D}$ expression eversed

complex genomic landscape is undoubtedly limiting our capability to act therapeutically to block the progression of the disease. Therefore, understanding the contribution of each genetic alteration to the pancreatic cancer development is of paramount importance to improve diagnosis and therapy.

The generation of mouse strains harboring the components of DNA transposon system have permitted in vivo insertional mutagenesis screens that have led to the identification of a plethora of novel cancer driver genes [66–69]. Transposon-based GEMMs harbor a transposon concatemer as the primary source of transposons, and a transposase to mobilize the transposons within the host genome. Importantly, transposon-based mutagenesis systems in mice have provided a valuable tool to reveal molecular pathways involved in different stages of PDA development in an unbiased manner.

Sleeping Beauty (SB) and *piggyBac* (PB) transposons are DNA transposable elements flanked by inverted repeat/direct repeat (IR/DR) sequences. Transposons harbor a strong promoter regulatory element to ectopically activate the expression of potential proto-oncogenes or dominant-negative tumor suppressor genes (gain-of-function activity), and bidirectional polyadenylation signals to trap upstream exons and inactivate potential tumor suppressor genes (loss-of-function activity) (Fig. 4). The mobilization of the transposons along the genome is mediated, using a non-replicative "cut-and-paste" mechanism, by site-specific transposases that specifically recognize the inverted terminal repeats. When transposons integrate in the genome, they regulate gene expression nearby the integration site [66].

In the last few years, transposon-based GEMMs have been used to identify new genes and molecular pathways that cooperate with $Kras^{G12D}$ during PanIN to PDA progression [19, 20, 70]. We generated a *Sleeping Beauty* (SB) conditional *knock-in* mouse model, *Rosa26-LSL-SB13*, that harbors a hyperactive *SB13* transposase under the control of the *Rosa26* promoter. Upstream of the *SB13* cDNA, a floxed transcriptional stop cassette (LoxP-STOP-LoxP, LSL) allowed the spatiotemporal control of *SB13* expression. The LSL cassette was removed in the pancreas by *Pdx1*-driven *Cre* recombinase expression (Fig. 4). We monitored a cohort of *LSL-*$Kras^{G12D/+}$*; Pdx1-Cre; T2/Onc; Rosa26-LSL-SB13* (*KCTSB13*) mice and found that SB13-mediated mobilization of a *T2/Onc* transposon concatemer in pancreatic progenitor cells led to a dramatic decrease in median survival due to the development of PDA and invasive cystic neoplasias (172 vs. 257 days of mice that do not harbor the *T2/Onc* and/or *Rosa26-LSL-SB13* alleles). The analysis of common insertion sites of the transposon in tumors obtained from *KCTSB13* mice revealed genes previously associated with human PDA including $p16^{Ink4a}$, *Rb*, components of the TGFβ signaling pathway, *Acvr1b, Arid1a, Stk11,* or *Pten*, confirming the biological significance of this approach. Additionally, the screen provided novel information of molecular pathways involved in PDA progression. The deubiquitinase *Usp9x* was the gene most commonly found to be inactivated in this screen, with over 50% of the KCTSB13 tumors (101 out of 198) showing inactivation of the *Usp9x* locus. Strikingly, we confirmed using in vitro approaches that *Usp9x* downregulation enhanced transformation of PDA cells. Furthermore, we generated *LSL-*$Kras^{G12D/+}$*;* $Usp9x^{flox/flox}$*; Pdx1-Cre* mice and found that *Usp9x* inactivation in the pancreas

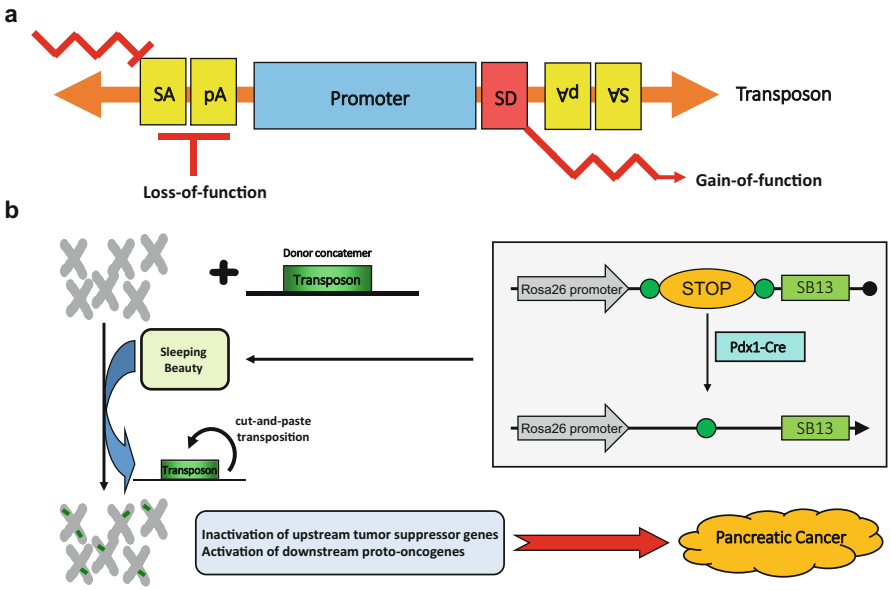

Fig. 4 Insertional mutagenesis screen in PDA. (**a**) Transposons harbor bidirectional polyadenylation sequences to trap upstream exons and inactivate potential tumor suppressor genes (loss-of-function activity), and a strong promoter to activate potential proto-oncogenes downstream of the integration site (gain-of-function activity). (**b**) *Pdx1-Cre*-mediated expression of *SB13* induces the mobilization of a transposon concatemer in the pancreas compartment leading to mutations that accelerate PDA progression

cooperated with $Kras^{G12D}$ to accelerate pancreatic cancer development. Significantly, data found in these GEMMs were corroborated clinically; *USP9x* was found to be downregulated in human PDA, and low *USP9X* levels correlated with poor prognosis and higher metastatic burden [19]. Overall, this study identified a novel tumor suppressor gene with prognostic and therapeutic relevance in PDA. Significantly, an independent study performed by Mann et al. [20] confirmed these findings. Using a similar approach, they found that SB11-mediated mobilization of the *T2/Onc2* or *T2/Onc3* transposon concatemers produced similar outcomes, a dramatic acceleration in pancreatic cancer progression and recurrent inactivation of *Pten* and *Usp9x* [20]. Strikingly, these two SB-mediated insertional mutagenesis screens unveiled mutations in axon guidance regulators, giving unbiased genetic evidence of the critical role of the SLIT/ROBO signaling in PDA development and supporting the genomic studies performed in clinical samples [18].

Finally, Rad et al. [70] performed a PB-mediated insertional mutagenesis screen in the pancreas. The authors generated *LSL-*$Kras^{G12D/+}$*; Pdx1-Cre; ATP1-S2; Rosa26-LSL-PB* mice, where the *ATP1-S2* transposon concatemer was mobilized by the PB transposase. These mice presented accelerated PanIN to PDA progression, with the development of classical pancreatic ductal adenocarcinomas and adenocarcinomas with hepatoid differentiation. The analysis of genes mutated by

PB transposons revealed novel driver candidates involved in pancreatic cancer progression, including gain-of-function mutations in *FoxP1* and *Fign* [70]. Remarkably, the top candidate genes diverged between the SB and PB screens. This was most likely due to the different integration preferences of SB and PB transposons, and confirmed that both transposon systems are complementary to study the pathogenesis of cancer.

Collectively, forward genetic screens have provided a valuable tool to identify mutations that promote pancreatic cancer progression, permitting an unbiased selection of pancreatic cancer driver genes among the candidate genes identified in genomic studies performed in human specimens. Additionally, these successful approaches support the use of GEMMs of pancreatic cancer to complement clinical studies and warrant the employment of transposon-based GEMMs for the identification of molecular mechanisms involved in drug resistance.

Mouse Models as a Tool to Develop Therapeutic Strategies to Fight Pancreatic Cancer: Understanding the Role of the Stroma in Chemoresistance

As described above, a collection of GEMMs of pancreatic cancer carrying diverse combinations of mutations have been generated. Importantly, the targeting of different molecular pathways has resulted in the development of tumors with distinct clinical and histopathological features that have led to a better understanding of the biology of pancreatic cancer. Taking advantage of this ability to recapitulate key clinical features of the human disease, GEMMs have been employed as a preclinical platform to develop new therapeutic intervention strategies and to understand the mechanisms of chemoresistance.

The use of mouse models of pancreatic cancer for preclinical studies was pioneered by the group of David Tuveson. They extensively employed the *LSL-Kras*$^{G12D/+}$*; LSL-Trp53*$^{R172H/+}$; *Pdx1-cre* (*KPC*) model that was shown to tightly recapitulate the biology of human PDA including an abundant desmoplastic stromal reaction associated with poorly vascularized tumors [71]. Supportively, *KPC* mice were found to be highly resistant to treatment with gemcitabine, mimicking human PDA chemoresistance and suggesting that the hypovascular and desmoplastic nature of PDA may be critical determinants for therapeutic response [71]. This hypothesis has been successfully proved by a number of studies that have used the *KPC* model as a preclinical model to evaluate how the depletion of the stroma impacts therapeutic response. Olive et al. [71] found that stroma depletion in established PDA using IPI-926, an inhibitor of the Hedgehog pathway effector Smoothened, led to an increase in tumor vascularization, enhanced gemcitabine delivery, and extended survival, suggesting that the tumoral stroma was a barrier for efficient drug delivery [71]. Discouragingly and surprisingly in view of the promising data from a Phase 1b trial, a Phase II trial showed a reduced median survival in patients on the gemcitabine + IPI-926 arm versus patients on the gemcitabine arm. This disappointing outcome

indicates that careful analyses are required to understand how GEMMs can be maximized for preclinical studies.

Interestingly, two recent reports have introduced controversies about the role of the stroma in PDA development. In the first study, Ozdemir et al. [72] ablated α-SMA+ myofibroblasts using an elegant genetic approach. They generated *LSL-Kras*$^{G12D/+}$*; Tgfbr2*$^{flox/flox}$*; Ptf1a/P48-Cre; α-SMA-tk*, and *LSL-Kras*$^{G12D/+}$*; LSL-Trp53*$^{R172H/+}$*; Pdx1-cre; α-SMA-tk* mice. Both cohorts expressed thymidine kinase (tk), an enzyme that transforms ganciclovir into a toxic product that selectively kills cells expressing *tk*, in α-SMA+ cells. Unexpectedly, myofibroblast ablation after treatment with ganciclovir led to the development of undifferentiated and invasive tumors and reduced overall survival compared with mice that did not receive ganciclovir. Importantly, these findings were supported by the analysis of clinical specimens. Indeed, patients with PDA and reduced numbers of myofibroblasts exhibited a shorter median survival. In line with these findings, Rhim et al. [73] deleted Shh in the pancreas compartment by generating *Shh*$^{flox/flox}$; *LSL-Kras*$^{G12D/+}$*; Trp53*$^{flox/+}$; *Pdx1-cre; LSL-Rosa26-YFP* (*ShhKP*$^{flox/+}$*CY*) mice. Significantly, *ShhKP*$^{flox/+}$*CY* mice showed accelerated tumor development compared with *KP*$^{flox/+}$*CY* mice. Furthermore, tumors displayed increased metastatic potential and exhibited a poorly differentiated histology accompanied by reduced stromal desmoplasia and increased angiogenesis. Significantly, this phenotype was recapitulated after chronic Smoothened inhibition using IPI-926. Taken together, these studies strongly suggested that at least some component of the stroma may be stopping tumor spread.

A number of additional studies have assessed different therapeutic approaches to target the PDA stroma. Two different groups have used PEGylated human recombinant PH20 hyaluronidase (PEGPH20) to evaluate the effect of targeting Hyaluronic Acid (HA), one of the major component of the extracellular matrix [74, 75]. PEGPH20 treatment reduced the interstitial fluid pressure generated by the high desmoplastic reaction and reexpanded the tumor vasculature, thereby increasing the delivery of gemcitabine. Importantly, PEGPH20 and gemcitabine combination nearly doubled overall survival of *KPC* mice over gemcitabine monotherapy. Significantly, a Phase Ib study has revealed that PEGPH20, in combination with gemcitabine, may benefit patients with advanced PDA, predominantly those with high HA tumors [76].

KPC mice have also helped to understand the antitumor activity of the nanoparticle albumin-bound (nab)-paclitaxel, which binds to SPARC (secreted protein acidic and rich cysteine) to promote stromal depletion. In immunodeficient mice with human pancreatic cancer xenografts, the nab-paclitaxel plus gemcitabine combination reduced tumor stroma, induced angiogeneisis, and increased intratumoral gemcitabine levels after 28 days of treatment [77]. A subsequent study by Frese et al. [78] showed that nab-paclitaxel and gemcitabine led to tumor regression and metastasis reduction in *KPC* mice after 8 days of treatment. Mechanistically, it was shown that nab-paclitaxel elevated intratumoral levels of gemcitabine through decreasing the levels of the gemcitabine-metabolizing enzyme cytidine deaminase, which resulted in the enhanced stabilization of gemcitabine. However, they did not

observe the depletion of stroma, likely due to the unfeasibility to treat *KPC* mice for 28 days due to the development of an acquired immune response to the human albumin component of nab-paclitaxel.

Another significant study showed that the tumor stroma limits antitumor immune response. Beatty et al. [79] showed that the combination of gemcitabine plus an agonist CD40 antibody, which activates T cell immunity, exhibited tumor regression in some patients with surgically incurable PDA. Interestingly, this treatment was recapitulated in *KPC* mice, although in this system the antitumor effect was not mediated by T cells, but on the CD40-activated macrophages, which promoted the depletion of tumor stroma [79].

Very recently, a significant study has shown that fibroblast drug scavenging may contribute to the clinical failure of gemcitabine in desmoplastic PDA. Hessmann et al. [80] showed that gemcitabine accumulation was considerably augmented in fibroblast-rich tumors. Importantly, primary PDA tumors show an increased number of α-SMA+ cells compared with matched liver metastases, suggesting that cancer-associated fibroblast (CAFs) may accumulate active gemcitabine intracellularly thus limiting the availability of the drug for cancer cells. Notably, gemcitabine treatment in KPC mice, although this did not extend overall survival, strongly reduced the number of liver metastases. Mechanistically, the authors demonstrated that metabolic enzymes involved in gemcitabine inactivation, including Nt5c1A and Nt5c3, were expressed at low levels in CAFs. Overall, this study unveils the metabolic targeting of CAFs as a potential promising strategy to enhance the antitumor activity of gemcitabine.

Collectively, preclinical studies using GEMMs of PDA have supported the development of novel therapeutic intervention strategies and also provided insights for understanding the molecular and cellular basis of chemoresistance in PDA. Information on additional preclinical studies can be found in [81, 82].

Conclusion

The improvement of gene-targeting approaches together with an increased understanding of the molecular basis of pancreatic cancer have led to the generation of GEMMs that faithfully reproduce the biology and histological evolution of pancreatic cancer. GEMMs of pancreatic cancer have been used not only to understand the molecular and cellular basis underlying pancreatic tumorigenesis, but also to unveil mechanisms of chemoresistance that have led to better therapeutic strategies. Nevertheless, current GEMMs have some limitations, and new approaches to modeling pancreatic cancer in mice are being developed.

The most accepted model for human pancreatic cancer development follows a stepwise genetic progression beginning with the activation of KRAS followed by the sequential inactivation of tumor suppressors during the progression of the disease [3, 4] (Fig. 1). Current mouse models are based on the use of a single recombinase, the Cre-LoxP or Flp-FRT systems. Accordingly, in these single recombinase-based GEMMs the activation of KRAS and inactivation of tumor suppressor genes happen

simultaneously in the same cell type, precluding the recapitulation of the genetic PDA progression model. Recently, Dieter Saur's laboratory have generated a dual-recombinase model for time- and host-specific targeting of pancreatic cancer by combining the Cre-LoxP and Flp-FRT systems, in which oncogenic Kras activation is mediated by the Flp-FRT system while the Cre-LoxP system enables the spatio-temporal regulation of a second genetic event [83]. This genetic approach permits a sequential genetic manipulation, facilitating the analysis of cooperating genetic events during PanIN to PDA progression, selective targeting of specific components of the tumor microenvironment, and genetic validation of therapeutic targets [83].

Current approaches to generate complex compound mutant strains are based on crossing individually targeted strains, which is extremely slow and highly costly. Therefore, new methods for a rapid generation of tailored GEMMs are greatly required. During the last few years, several approaches have been developed to accelerate in vivo studies:

1. Saborowski et al. [84] developed an embryonic stem cell (ESC)-based GEMM system to generate multiallelic chimeric mice. They established 2 ESC lines harboring four mutant alleles. Firstly, *LSL-Kras*$^{G12D/+}$ and *Pdx1-Cre* (or *Ptf1a/P48-Cre*) alleles to initiate PDA. Additionally, a recombinase-mediated cassette exchange (RMCE) targeted in the *col1a1* locus to facilitate high-efficiency targeting with tetracycline-regulatable shRNAs or cDNAs. Finally, a *CAGs-LSL-rtTA3-IRES-mKate2* allele drives *Cre*-mediated *rtTA3* (tetracycline trans-activator) and fluorescent *mKate2* protein expression in pancreatic progenitor cells. When doxycycline is administrated, rtTA3 induces the expression of the shRNA or cDNA cloned in the *col1a1-RMCE* allele. As proof of concept, the authors showed that *Pten* knock-down cooperated with *Kras*^{G12D} to accelerate PDA development, while *c-Myc* downregulation compromised PDA development.
2. Another approach was developed by Dieter Saur's laboratory [85]. They used RCAS-TVA-mediated retroviral gene transfer to downregulate or overexpress target genes in pancreatic cells that express the retroviral receptor TVA. They generated *LSL-Kras*$^{G12D/+}$*; Ptf1a/P48-Cre; Rosa26-LSL-TVA-lacZ* mice to direct TVA expression to pancreatic cells after Cre-mediated excision of the LSL cassette. TVA-mediated infection of pancreatic cells expressing *Kras*^{G12D} with retroviruses carrying a shRNA against *Trp53* dramatically enhanced PDA development, confirming that this system can be used to target neoplastic cells in vivo.
3. Finally, two groups have showed that CRISPR/Cas9 technology can be used to study cooperating events in PDA. Chiou et al. [86] showed that the lentiviral delivery of *Cre* recombinase and *sgLkb1* in pancreata of *LSL-Kras*$^{G12D/+}$*; Rosa26-LSL-Tomato; H11-LSL-Cas9* mice led to PDA development associated with *Kras*^{G12D} activation and *Lkb1* ablation. A second group showed that transfection-based multiplexed delivery of CRISPR/Cas9 to the pancreata of *LSL-Kras*$^{G12D/+}$*; Ptf1a/P48-Cre* mice permits important applications, including combinatorial gene-network analysis, synthetic lethality screening, and chromosome engineering [87].

In conclusion, GEMMs have provided an invaluable tool for understanding pancreatic cancer pathogenesis, complementing clinical studies and improving our capability to develop new therapeutic intervention strategies. It is expected that refinement of the current GEMMs will provide new avenues to enhance our knowledge of this lethal disease.

Cross-References

- ▶ Animal Modeling of Pancreatitis-to-Cancer Progression
- ▶ Apoptosis: Signaling Pathways in Pancreatic Cancer Pathogenesis
- ▶ EGFR (ErbB) Signaling Pathways in Pancreatic Cancer Pathogenesis
- ▶ Hedgehog Signaling Plays a Dual Role in Pancreatic Carcinogenesis
- ▶ Notch Signaling in Pancreatic Morphogenesis and Pancreatic Cancer Pathogenesis
- ▶ Role of Tumor-Stromal Interactions in Pancreatic Cancer Invasion and Metastases
- ▶ Smad4-TGF-β Signaling Pathways in Pancreatic Cancer Pathogenesis
- ▶ The Molecular Pathology of Precursor Lesions of Pancreatic Cancer

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Role of Tumor-Stromal Interactions in Pancreatic Cancer Invasion and Metastases

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Abstract

Pancreatic cancer tumor microenvironment (TME), simply defined as the non-cancerous desmoplastic reaction, is considered a key player in all aspects of tumor growth, and progression. The dismal prognosis of pancreatic cancer and disappointing clinical trials has drawn our attention to the TME, particularly to the tumor-stromal interactions. While a myriad of molecular, pathological, and clinical features contribute to the lethality of pancreatic cancer, local invasiveness and distant metastases is a hallmark and leading cause of mortality and morbidity in this ominous cancer. Cancer-associated stromal cells including stellate cells have been implicated in epithelial mesenchymal transition (EMT), a process involved in invasion and metastases. In addition, the pre-metastatic niche, immune evasion, and enhancement of angiogenesis have been attributed to these cells. Interactions of the tumor stromal complex operate as a command and logistics center for pancreatic cancer cells, triggering and maintaining invasiveness and metastases. Understanding and modulating these interactions is a promising strategy to tame one of the most aggressive human cancers to date.

Keywords

Pancreatic cancer · Tumor microenvironment · Cancer-associated fibroblast · Pancreatic cancer stellate cells · Tumor-cell interaction · Metastasis

Introduction

Pancreatic cancer (PC) is the third leading cause of cancer related-deaths in the world and is predicted to be second by 2030 [1]. It is an early asymptomatic and aggressive disease with a 5-year survival rate of 8% [2]. Upon diagnosis, only 10–15% are resectable with the remaining being metastatic [3]. Even in the completely resected primary tumor, the cause of death is local or systemic recurrence [4, 5]. The dismal prognoses of looming mortality is due to the propensity for early metastatic spread coupled to ineffective treatments [6]. Improvements to surgical resection are ongoing, and neoadjuvant and adjuvant systemic therapy and antimetastatic agents are gaining popularity. Even with these enhancements, metastasis in pancreatic cancer is inevitable [6]. Pancreatic cancer is characterized by its tumor microenvironment (TME) and its implication in tumor progression [7, 8]. The cellular environment and all of its components for which the tumor exists collaboratively promotes primary tumor growth and metastasis [9, 10]. The cellular and noncellular components of pancreatic cancer can drive host immune evasion, epithelial to mesenchymal transition, and invasiveness [11–13]. Moreover, they can be responsible for the resistance to chemotherapy and radiotherapy of pancreatic tumoral cells [14, 15].

Cellular and Noncellular Components of Pancreatic Tumor Microenvironment

Pancreatic cancer is not merely an isolated mass of malignant cells but a complex interaction of different cell types and noncellular elements [16, 17]. The cellular components of pancreatic TME are numerous. Of these components, stroma may account for greater than 80% of the total tumor volume of which cancer-associated fibroblasts (CAFs) are the most numerous stromal cell together with pancreatic stellate cells (PSC) [9, 18]. These secretory cells promote tumor growth and proliferation; modulate cancer cell metabolism, immunosuppression, extracellular matrix (ECM) remodeling; and increase metastatic processes [18, 19]. The phenotypic and functional heterogeneity observed in CAFs can be partially explained through their diverse cellular origins ranging from resident tissue mesenchymal cells (e.g., pancreatic stellate cells), bone marrow-derived mesenchymal stem cells, hematopoietic stem cells to epithelial and endothelial cells [20, 21] (Fig. 1). Resting PSC account for 4% of the normal pancreatic tissue and are spindle-shaped cells with a prominent rough endoplasmic reticulum, collage fibrils, and lipid droplets with expressions of desmin and glial fibrillary acid protein (GFAP) and vimentin on the cytoplasmic membrane [8]. Upon injury and inflammation the resting PSC loses its vitamin A storage and acquires a star-shaped morphology, expresses α-SMA, migrates and proliferates while secreting copious amounts of ECM, growth factors, and cytokines [8, 16]. Because of this, activated PSCs are often considered the "architect" cells of PC stroma and are an attractive therapeutic target and will be the focus of the next section.

Pancreatic tumors contain copious immune cells, yet are frequently immunosuppressive [22]. PC tumors are T-lymphocyte rich; CD8+ T-cells are associated with a promising prognosis whereas T-regulatory (Treg) cells are immunosuppressive and favor tumor growth [12, 23]. In advanced stages of PC B-lymphocytes have been found to promote cancer through polarization and immunosuppression of macrophage activity. Macrophage recruitment promotes angiogenesis, immunosuppression, and ECM remodeling enhancing tumorogenesis [24]. Myeloid-derived suppresser cells (MDSCs) are recruited in mass and inhibit the activity of T-cells in the pancreatic cancer TME [25, 26]. The immune-suppressive environment of PC serves as potential therapeutic targets and is influenced through communication with neighboring stromal and tumorgenic cells.

The noncellular components of the TME typically refers to the makeup of the extracellular matrix (ECM). Here, the ECM not only serves as a scaffold to house cellular components but functions in the evolution and metastasis of pancreatic cancer. For example, secreted protein acidic and rich in cysteine (SPARC) is a major noncellular element of the ECM in remodeling tissues and enhances intratumoral drug delivery [22]. Other elements such as tenascin C, periostin, and proteases contribute to enhanced tumor proliferation, aggressiveness, invasiveness, and migration whereas osteopontin stromal content correlates with better survival

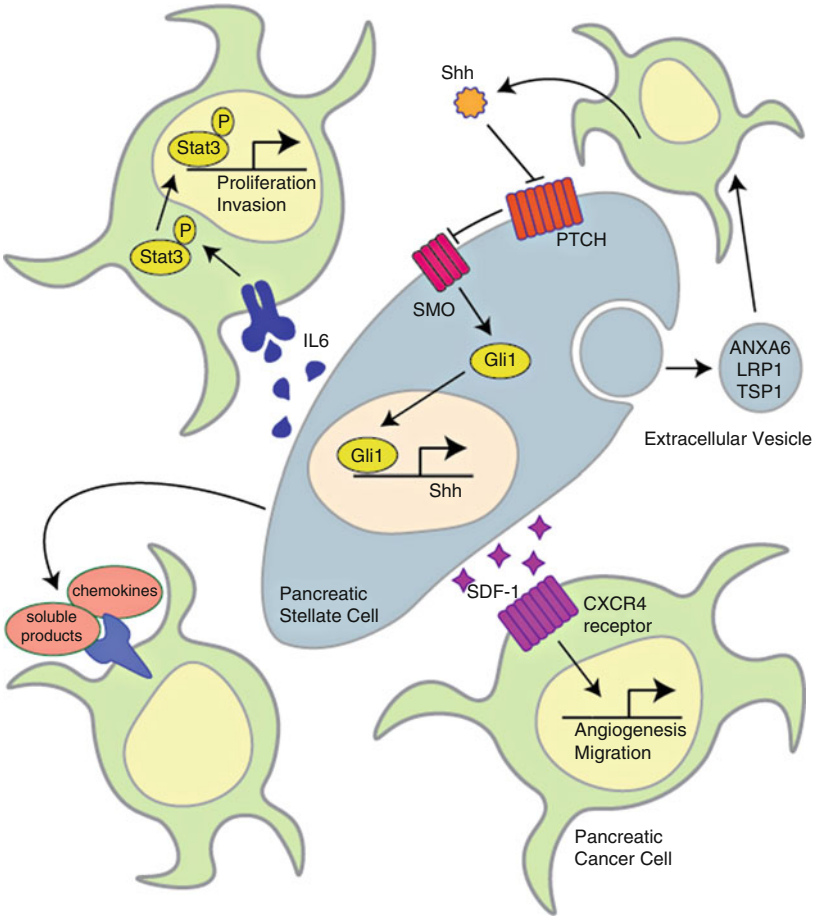

Fig. 1 Cancer-stroma cross-talk involves cellular communication between the tumor and the cellular environment for which the tumor exists. Activated PSCs are implicated in most all PCC processes and secrete copious amounts of IL-6 which activates STAT3 signaling in PCCs and promotes tumor cell proliferation and invasion. SDF-1 expression from PSCs promotes tumor angiogenesis and migration through the SDF-1/CXCR4 receptor ligand axis. Extracellular vesicle proteins ANXA6, LRP1, and TSP1 are secreted from PSCs and alter TME conditions, enhance PC invasion, survival, and aggressiveness. Various interleukins involved in paracrine signaling including chemokines and soluble products are secreted into the ECM from PSCs and act as mediators for tumorigenic invasion. PSC activation is enhanced through PCC secretion of Hedgehog and subsequent transcriptional activation mediated through GLI family transcription factors; overexpression of GLI1 and Hedgehog proteins are closely associated with PC. These interactions of the tumor-stromal complex initiate and maintain invasiveness and metastases of pancreatic cancer

rates [22]. The ECM serves as a conduit for communication between pancreatic cancer cells (PCCs) and noncancer cells and contributes to metastatic properties. PCCs alter the extracellular matrix through asserting direction via secretion of growth factors and chemokines into the ECM [27].

Due to the importance of the extracellular matrix (ECM) in cell behavior any alteration in the composition or structure of the ECM network can alter tissue architecture promoting the loss of normal function generating diseases, such as cancer [28]. The extracellular matrix is a noncellular well-organized network in which cells reside. In the pancreas the ECM influences a wide range of cellular processes; these include pancreatic islet cell development, survival, proliferation, and differentiation, as well as β-cells insulin secretion. Pancreatic human extracellular matrix consists of two parts: basement membranes (BM), which are found closely associated to islet cells, and an external thin layered interstitial matrix. The basement membrane, also known as peri-islet BM, is principally formed by collagens and layers of laminins (LN) [29]. Collagens provide a scaffold for embedded cells while laminins are essential to promote signal transduction mediated by interactions with cell surface receptors such as integrins. Basement membranes also contain fibronectin (FN) [30]. Heparin sulfate (HS) is a negatively charged glycosaminoglycans (GAGs) attached to core proteins, and favors bindings with different molecules including cytokines, growth factors, and chemokines [31]. Through these interactions signal transduction is promoted which leads to cell behavior control. Cells interact with ECM components through their surface receptors: integrins, discoidin domain receptors (DDRs), transmembrane proteoglycans such as syndecans, and the hyaluroan (HA) receptor CD44 [30]. Here the ECM influences cell behavior and gene expression through specific signal transduction as well as changes in interstitial fluid pressure which can be a barrier to perfusion, diffusion, and convection of small molecules therapeutics.

Tumor-Stromal Interactions in Pancreatic Cancer

Cancer-stroma "cross-talk" involves cellular communication between the tumor and the cellular environment for which the tumor exists [32]. For example, activated PSCs are implicated in a large number of PCC processes including intracellular signaling, carcinogenesis, growth, induction of EMT, invasion, migration, metastases, and even therapeutic resistance [33]. The molecular pathways that drive pancreatic cancer are comprised of oncogenes, tumor suppressor genes, and developmental signaling pathways [34]. These molecular alterations show varying incidence and exhibit a temporal order with cancer progression and correlate with the morphological, histopathological, and clinical context [34, 35].

Pancreatic Cancer Cells Hijack Immune Cells

PCCs modulate the innate and adaptive immune system through recruitment and potentiation of immunosuppressive cells [36]. In many cases, PC is initiated by oncogenic KRAS, which has been shown to recruit macrophages in addition to driving neoplasia [37]. The classic antitumor role of the immune system is

represented by cytotoxic CD8+ and Th1 cells infiltrating the stroma, bone marrow, and blood [12]. The noncanonical role of the immune system enhances tumor survival. Activated PSC induce Stat-3 differentiation of myeloid-derived suppressor cells (MDSCs) [38], mast cell proliferation, T-cell apoptosis, and inhibition of further T-cell tumor infiltration [39, 40]. In the context of innate immunity, the most prominent immune cell associated with PC stroma is the tumor-associated macrophage (TAM) or CD11b + macrophage [22]. Tumor invasion is enhanced through TAM recruitment from blood monocytes or resident tissue macrophages which suppress the antitumor T-cell response [22]. PCCs mediate macrophage differentiation [41] and other tumor-infiltrating immune cells to promote cancer growth and progression by TGF-β and IL-4 [42]. Macrophages chemo-protect PCCs through upregulation of enzymatic degradation of the chemotherapeutic agent gemcitabine and enhance tumor invasion [15, 41]. The PCC-PSC-mast cell communication recruit and activate mast cells, and reciprocally, mast cells contribute to stromal proliferation through IL-13 and tryptase resulting in cancer progression by TGF-β/Smad2 axis [43]. In addition, PCCs mobilize myeloid-derived suppressor cells (MDSCs) and mediate activation by Stat-3, and in return MDSC and other TME immune cells such as TAM [44] enhance the self-renewing therapy-resistant cancer stem cells (CSCs) [45].

L1CAM (CD171) an adhesion molecule, involved in the adaptive immune response, is overexpressed in PC and promotes the migration and infiltration of regulatory T cells (Treg cells) [36, 46]. PCCs stimulated by T cell gamma-interferon (INF) upregulate the immune inhibitory checkpoint PD-1 which reduces the late inflammatory reaction in the TME [47]. Contrary to this, CTLA-4 hinders the amplitude of early T cell activation [47]. Myeloid-derived suppressor cells (MDSC) infiltrate not only the tumor stroma but also bone marrow, the spleen, and blood stream; MDSCs induce regulatory Treg cells and attenuate antigen-specific T-cell response [22, 25]. In PC Treg cells are immunosuppressive through an induction of IL-10 and TGF-b blocking T-cell antigen responses [22]. These immune cell networks create an immune suppressive environment. Here, mast cells are recruited by PCCs and activated. Activation of mast cells enhance PSC proliferation and deposition of ECM [48]. Furthermore, activated PSC express Galectin-1, a β-galactoside binding lectin [49], that inhibits T cell activation, proliferation and promotes T cell apoptosis [50]. Knocking down Galectin-1 boosted the viability of CD4+ and CD8+ T cells [39, 50]. Targeting of Galectin-1 and PSC-IL-6-Stat3 pathway could neutralize the PSC-mediated immunosuppression [50, 51]. PC is immunotherapy resistant whereas other immune-active tumors such as melanoma are effectively treated with single immune-therapeutics targeting immune-inhibitory checkpoints such as anti-PD1 and anti-CTLA-4 [52, 53]. Converting PC to an immunogenic tumor is the key to overcoming this immunotherapy resistance [54]. Preclinical and clinical trials using combination immune therapy including a cancer vaccine and an immune checkpoint inhibitor have shown synergism [47, 54].

Stromal Cells Promote PCC Epithelial-Mesenchymal Transition

Cellular plasticity gained through epithelial-mesenchymal transition (EMT) contributes to stress adaptation and facilitates cancer progression and dissemination [6, 55]. EMT involves molecular processes engaged in reprogramming phenotypic and functional epithelial cells into motile and supportive mesenchymal cells [55]. The trigger(s) of EMT in PC remains elusive, yet, a myriad of growth factors, cytokines, intracellular pathways, and epigenetic cascades are known to participate in this process [56]. PSCs have been implicated in the promotion of EMT through modulation of the levels of mesenchymal genes including vimentin, Snail, and beta-catenin in PCCs morphology and enhances expression of mesenchymal markers vimentin, Snail, and beta-catenin [57, 58] Inflammation-healing are linked to EMT through molecular processes involving the activation of Notch, Hedgehog-GLI, TGF-β, and PDGF signaling (Fig. 1) [13, 14, 59, 60].

Epithelial cells metamorphosing into phenotypic and functional mesenchymal cells is characterized by the loss of E-cadherin and acquisition of N-cadherin and vimentin, markers of epithelial and mesenchymal cells, respectively [61]. EMT associates with cytoskeletal alternation, basement membrane invasion, venous infiltration, nodal metastases, and poor survival [61–63]. PCCs undergoing EMT have stem-cell-like properties and are integral in the development of metastatic PC [64, 65]. Circulating mesenchymal cells in blood of pancreatic cancer patients due to EMT are detectable prior to primary tumor diagnosis [64]. These findings indicate PC development is associated with EMT and that within the molecular pathways governing EMT there exists potential therapeutic targets. Of note, CAF/PSC-induced EMT is inhibited by retinoic acid through suppression of IL-6 secretion, and thus representing a novel therapeutic target for the treatment of advanced PC [66].

The TGF-β signaling pathway is implicated in epithelial cell arrest and tumor suppression [67]. However, it can also promote tumor growth by inducing EMT. For examples, TGF-β ligand is most abundant in PC stroma where it activates PSCs and upregulates ECM proteins fibronectin and collagen type I [68]. The role of TGF-β as guardian turned aggressor is in part explained by the dual function of the pathway and the imbalance between SMAD4 dependent and independent TGF-β pathways [67]. Loss of SMAD4 and consequent loss of the SMAD4-dependent TGF-β signaling abolishes tumor suppressor function of TGF-β. SMAD4 loss has been correlated with mesenchyme histological features, portal vein, lymph vessel, and perineural invasion as well as disease-specific and disease-free survivals [69]. SMAD4, a tumor suppressor molecule, mediates the shift of TGF-β function from tumor prevention to tumor promotion via boosting its invasive and metastatic potentials [67, 70]. In addition, an antibody blockade of TGFβ not only modulates tumor stroma to a less immunosuppressive and more antitumor profile but results in a greater epithelial phenotype with less metastatic potential [71]. In contrast, intact SMAD4 in PCCs correlate with TGF-β related proliferation, indicating that wild-type SMAD4 can be more responsive to TGFβ inhibition [71]. The overexpression

of TGF-β in pancreatic cancer skews the balance towards tumor promotion via the SMAD4-independent pathways as PI3KAKT, ERK, and p38 MAPK, NFκB/PTEN and STAT3 [67]. Together, the pro-cancer and pro-metastatic role of TGF-β late in the disease context suggests that SMAD4 status may be used to segregate the patient group that may benefit from TGF-β blockade.

Pancreatic Stellate Cells Enhance Pancreatic Cancer Invasiveness

PSCs boost PCCs infiltrative affinity by at least three distinct avenues. PSCs secrete various interleukins involved in paracrine signaling including chemokines and soluble products which act as mediators for invasion. Importantly, activation of IL-6 with its downstream signaling mediator STAT3 promote intraepithelial neoplasia to invasive cancer (Fig. 1) [72–74]. In vitro studies demonstrate that PCC invasiveness was influenced by expression of CCR9 and DSF1 expression through contributing to diminished cell-to-cell contact [75, 76]. SDF-1 belongs to the CXC chemotactic family that is related to the SDF-1/CXCR4 axis, which is of paramount importance to the mechanism and prevention of HIV-1 infection (Fig. 1). This suggests a metastatic potential in other solid cancers such as breast, lung, prostate, ovarian, and stomach cancers [76]. Of interest, PSCs and not PCCs express SDF-1 which activate the CXCR4 axis in cancer cells promoting migration and invasion. Moreover, PSCs supernatant enhances the migration of cancer cells in a dose-dependent manner through collagen-I the most abundant ECM component [76].

Extracellular vesicles (EV) have been identified to influence PSC/CAF-related cancer invasion [38, 63, 77]. Eleven stromal-specific proteins were identified to form a complex and may play a role in PC invasion. Among these proteins, ANXA6, LRP1, and TSP1 are of interest as they are associated with membrane-related events and cell-to-cell contact [31, 77]. These three proteins are secreted from PSCs/CAFs and alter TME conditions including hypoxia, lipid deprivation, and macrophage presence (TAM). In addition, ANXA6, LRP1, and TSP1 are not only enclosed in extracellular vesicles but are imperative for PC invasion, survival, and aggressiveness [77]. Of interest, higher ANXA6 levels in circulating EVs were correlated with a higher tumor grade and detrimental survival. Based on that, ANXA6, LRP1, and TSP1 had a proven clinical utility suggested by its diagnostic and predictive value [77].

A typical feature of PC is its local tissue and vascular invasion with subsequent distant metastases. Matrix metalloproteinase-2 (MMP-2), an endopeptidase with proteolytic activity targeting the degradation of the basement membrane during EMT, is secreted from PC stroma and associated with vascular invasion and metastases [78, 79]. MMP-deficient PCCs shed their trans-membrane glycoprotein basigin (BSG) from their cell surface and stimulate the production of MMP from PSCs [78]. In vivo studies and examination of postoperative human species confirmed stromal overexpression of MMP-2 and MMP-6 and correlated that overexpression to the histologic invasion of large veins [80]. MMP-2 and MMP-6 have been shown to degrade collagen type IV, a major component of the venous basement membrane [80].

From this, PSCs secretion of MMPs offers a partial mechanistic explanation underlying tissue and vascular invasion. TGF-β a key signaling mediator involved in PC stroma, as well as IL-32α, hinders and reverses the invasive behavior of PC via counteracting the MMP secretory and invasive effects of IL-6/STAT-3 signaling and IL-1, respectively [81, 82]. Pancreatic stellate cells express pro-angiogenic factors such as VEGF, VEGF receptor, and angiopoietin-1 while also secreting anti-antiogenic factors vasohibin-1 and endostatin [39]. The role of PSCs in the pre-metastatic niche were found to migrate from the primary tumor to multiple metastatic sites in mice injected with male PSCs and female PCCs. These mice had enhanced angiogenesis via upregulation of the endothelial cell marker CD31 [83], and PSC migration is controlled via calcium-sensitive potassium channels (Kca3.1) along with other cytoskeletal and cell adhesion dynamics [84].

Conclusions

PC is a dismal prognosis due to the propensity for early and quick metastatic spread coupled with ineffective treatments. The cellular environment and all of the components for which the pancreatic tumor exists collaboratively determine tumor growth. Interactions between cancerous cells, noncancerous cells, and noncellular components comprise the TME. Activated PSCs are implicated in most all PCC processes and tumorigenesis. Prominent secretory cancer-associated fibroblasts promote tumor growth and proliferation through modulation of metabolism, ECM structure, and immunosuppression. Tumor cells manipulate and evade antitumor immunity while cellular plasticity through EMT facilitates cancer progression and metastasis. PSCs boost PCC metastatic potential through paracrine signaling and EV protein trafficking. In addition, PSCs augment PCC invasive nature directly through paracrine signaling and EV pathways. Through understanding the molecular pathways involved in PSC and PCC communication and deliberately targeting these interactions is a promising strategy to combat PC.

Cross-References

- ▶ Emerging Therapeutic Targets in Pancreatic Adenocarcinoma
- ▶ Epigenetic Pharmacology
- ▶ Epigenetics and Its Applications to the Progression Model of Pancreatic Cancer
- ▶ Hedgehog Signaling Plays a Dual Role in Pancreatic Carcinogenesis
- ▶ Molecular Pathology of Carcinomas of the Ampullary/Periampullary Region
- ▶ Molecular Pathology of Pancreatic Endocrine Tumors
- ▶ Pancreatic Cancer Stem Cells
- ▶ Smad4-TGF-β Signaling Pathways in Pancreatic Cancer Pathogenesis
- ▶ The Molecular Pathology of Precursor Lesions of Pancreatic Cancer

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Familial Pancreatic Cancer

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Abstract

Inherited genetic changes, from high-penetrance mutations to common genetic variants of modest effect, play a significant role in pancreatic cancer risk both in the familial and nonfamilial forms of the disease. Approximately 20% of the familial clustering of pancreatic cancer is explained by inherited mutations in *BRCA2*, *BRCA1*, *CDKN2A*, *PALB2*, *ATM*, *PRSS1*, *STK11*, *MLH1*, *MSH2*, *MHS6*, and *PMS2*. Even among families without an identifiable germline mutation, the presence of a family history of pancreatic cancer is a strong risk factor for the development of pancreatic cancer. Given the substantial increased risk of pancreatic cancer associated with a family history, many clinical trials aimed at the early detection of pancreatic cancer in this population are underway. The goal of this chapter is to review the evidence supporting the importance of a family history of pancreatic cancer as a risk factor for pancreatic cancer and the clinical and pathological features of familial pancreatic cancer.

Keywords

Familial pancreatic cancer · BRCA2 · ATM · Inherited susceptibility

Introduction

Pancreatic cancer is a devastating disease that affects over 200,000 people worldwide and approximately 50,000 people in the United States (USA) each year [1]. Patients with a diagnosis of pancreatic cancer often have a dismal prognosis. Between 1975 and 2013, the 5-year survival rate for pancreatic cancer has risen from 5% to 8%; however, pancreatic cancer still has the worst prognosis of any major tumor type (Fig. 1) [2].

Pancreatic cancer is a disease of increasing age, with a median age of onset of 71 years [1]. It is projected that pancreatic cancer will be become the second leading cause of cancer-related death in the United States by 2020 [3].

Familial Pancreatic Cancer (FPC)

The presence of a family history of pancreatic cancer is the strongest risk factor for the development of pancreatic cancer identified to date other than age [4, 5]. Up to 10% of pancreatic cancer cases report a history of pancreatic cancer in a close relative [6]. The current criteria for familial pancreatic cancer are the occurrence of two first-degree relatives (a parent and child or two siblings) with pancreatic ductal adenocarcinoma (PDAC) in a kindred [7]. While the occurrence of a familial clustering of pancreatic cancer can be due to an underlying genetic susceptibility, environmental risk factors, or stochastic effects, inherited genetic factors have been shown to play an important role, both due to high-penetrance gene mutations [8] and lower-penetrance common variants [9].

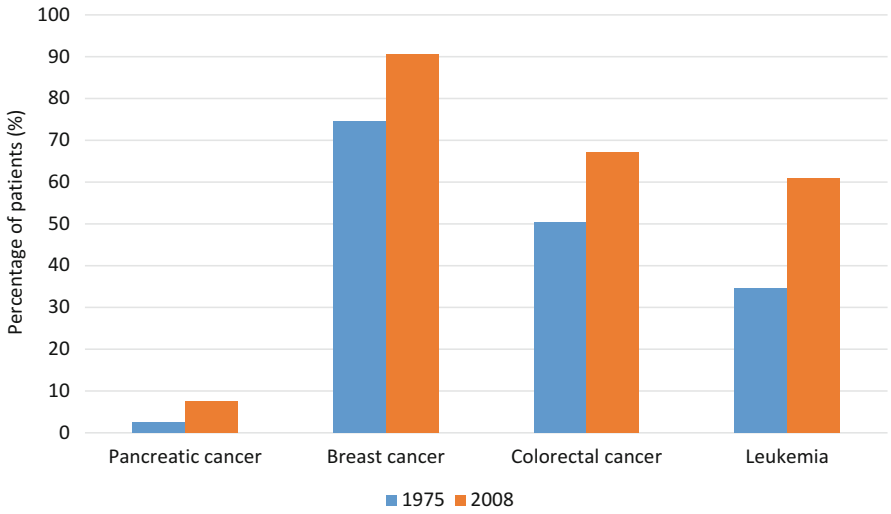

Fig 1 5-year survival rate for patients diagnosed with pancreatic cancer, breast cancer, colorectal cancer, and leukemia in 1975 and 2008. Graph compiled from the National Cancer Institute, Surveillance, Epidemiology, and End Results (SEER) Program data. Only females are included in breast cancer data [2]

The first reports in the literature of the clustering of pancreatic cancer in families were in the early 1970s. These include reports of multiple siblings with pancreatic cancer to small series of families with multiple pancreatic cancers. These initial case reports were followed by more rigorous controlled observational studies demonstrating increased risk of pancreatic cancer risk among individuals with a family history of the disease [10–18]. The risk estimates from these studies were highly variable, ranging from 1.5 to 13. However, a recent study, which pooled data from 1,183 cases and 1,205 controls with the Pancreatic Cancer Cohort Consortium, reported a multivariate-adjusted odds ratios (ORs) = 1.76, 95% confidence interval (CI) = 1.19–2.61 when comparing reported family history of pancreatic cancer in cases compared with controls [18]. However, the overall percentage of cases that reported a family history was quite low, and too few families met the criteria for familial pancreatic cancer to obtain a meaningful risk estimate in this group. In addition, due the nested case control design, family history may have been ascertained many years prior to the onset of pancreatic cancer in the cases, and the prevalence of a family history of pancreatic cancer at diagnosis may be higher than that reported [18]. In contrast to these population-based studies, numerous registries of familial pancreatic cancer kindred have been established in Europe, Japan, and the United States. One of the largest is the National Familial Pancreatic Tumor Registry at Johns Hopkins. Studies of incident pancreatic cancers that developed in at-risk family members who were disease-free when the families enrolled in the registry have shown that members of familial pancreatic cancer kindreds have at least a sevenfold increased risk of developing

pancreatic cancer [4, 5]. In contrast, individuals who had only a single relative with pancreatic cancer or multiple cases of pancreatic cancer in more distance relatives had about a 2.5-fold increased risk. Risk increases as the number of affected family members increases [4].

Familial pancreatic cancer is unlike inherited cancer syndromes where there is a strong association between family history and age of onset, with higher proportion of younger patients reporting a family history compared to older patients. Overall, the mean age of onset of pancreatic cancer in familial pancreatic cancer kindreds is at most only 6 years younger than the mean age of onset in those without a family history of pancreatic cancer, with many studies reporting no significant difference in age of onset between patients with and without a family history of pancreatic cancer [4, 16, 19, 20].

In addition to pancreatic cancer, other cancers occur more frequently than expected due to chance alone in familial pancreatic cancer kindreds. Relatives of patients with familial pancreatic cancer are also at an increased risk of dying from cancer at other sites including breast (weighted standardized mortality ratio (wSMR) 1.66, 95% CI 1.15–2.34), ovarian (wSMR 2.05, 95% CI 1.10–3.49), and bile duct cancers (wSMR 2.89, 95% CI 1.04–6.39) [21]. Mortality from cancer was elevated among relatives of all pancreatic cancer cases, both those who were members of familial pancreatic cancer kindreds (wSMR 1.41, 95% CI 1.26–1.58) and members of apparently sporadic pancreatic cancer kindreds (SMR 1.55, 95% confidence interval (95% CI) 1.39–1.73) [21].

While the increased risks described above could be attributed to shared environmental factors or genetic factors, both twin studies and segregation models support shared genetic factors as the basis of the clustering of pancreatic cancer in some families. Heritability estimates from twin studies suggest 36% (95% confidence interval 0.00–0.53) of the variability in pancreatic cancer is due to shared genetic effects [22]. Segregation analysis supported a dominantly inherited major gene(s) with a population prevalence of ~0.7% responsible for the clustering of pancreatic cancer in families. Lifetime risk in gene carriers was estimated to be 32% by age 85 [8].

Pathology of Familial Pancreatic Cancer

Oftentimes the cancers that arise in individuals with a hereditary cancer syndrome have a different pathological phenotype than cancers that develop in individual with a family history of cancer. For example, mismatch repair-deficient cancers are far more common among individuals with Lynch syndrome patients [23, 24] and triple-negative breast cancers are more common among *BRCA1* mutation carriers [25]. However, to date no study had identified a significant difference between pancreatic cancers that develop in individuals who report a family history of pancreatic cancer and the pancreatic cancers that develop among individuals

Table 1 Pancreatic Cancer Susceptibility Genes and associated extrapancreatic malignancies

Gene	Extrapancreatic malignancies/associated syndrome
ATM	
BRCA1	Breast cancer, ovarian cancer
BRCA2	Breast cancer, ovarian cancer
CDKN2A	Melanoma
Mismatch repair genes (MLH1, MSH2, MSH6, PMS2)	Colorectal cancer, endometrial cancer
PALB2	Breast cancer
PRSS1	–
STK11	Colorectal cancer

with no family history (apparently sporadic cancers). Recently a detailed review was conducted, blinded to family history, of 519 familial and 651 sporadic pancreatic cancers [26]. In this study no statistically significant differences between familial and apparently sporadic invasive pancreatic cancers in histologic subtypes were reported. When a focused analysis was conducted on early-stage cancers that underwent surgical resection, no significant differences in mean tumor size, location, angiolymphatic invasion, perineural invasion, lymph node metastasis, or pathologic stage were observed.

In addition to tissue studies, no significant differences have been observed between familial and apparently sporadic pancreatic cancers at the genetic level. The frequency of mutations in the established pancreatic cancer driver genes of *KRAS*, *P53*, *SMAD4*, and *CDKN2A* was quite similar [27]. However, examination of the pancreata adjacent to the pancreatic cancer in resected tissue samples from patients with both familial and sporadic pancreatic cancers reported that individuals with familial pancreatic had a significantly higher rate of PanIN per square centimeter 2.75 (95% CI, 2.05–3.70: adjusted for age) than patients with sporadic pancreatic cancer. In addition, familial pancreatic cancer patients had a higher rate of PanIN-3 lesions 4.20 (95% CI, 2.22–7.93) and high-grade IPMNs was were observed only in patients with familial pancreatic cancer [28]. Thus, the current data indicates that while the cancers that develop in patients with familial pancreatic cancer are similar histologically and genetically to the cancers that develop in patients with apparently sporadic disease, familial patients have more precursors and more advanced precursors than apparently sporadic patients.

In 15–20% of familial pancreatic cancer patients, susceptibility to pancreatic cancer can be attributed to deleterious germline variants in one of the 11 established familial pancreatic cancer susceptibility genes that include *ATM*, *BRCA1*, *BRCA2*, *CDKN2A*, *MLH1*, *MSH2*, *MSH6*, *PALB2*, *PMS2*, *PRSS1*, and *STK11* (Table 1) [29–31]. In the remaining 80–85% of familial pancreatic cancer patients, the underlying cause of disease susceptibility is unknown (Fig. 2). Therefore, there are likely unidentified susceptibility genes driving increased pancreatic cancer risk in these patients and families.

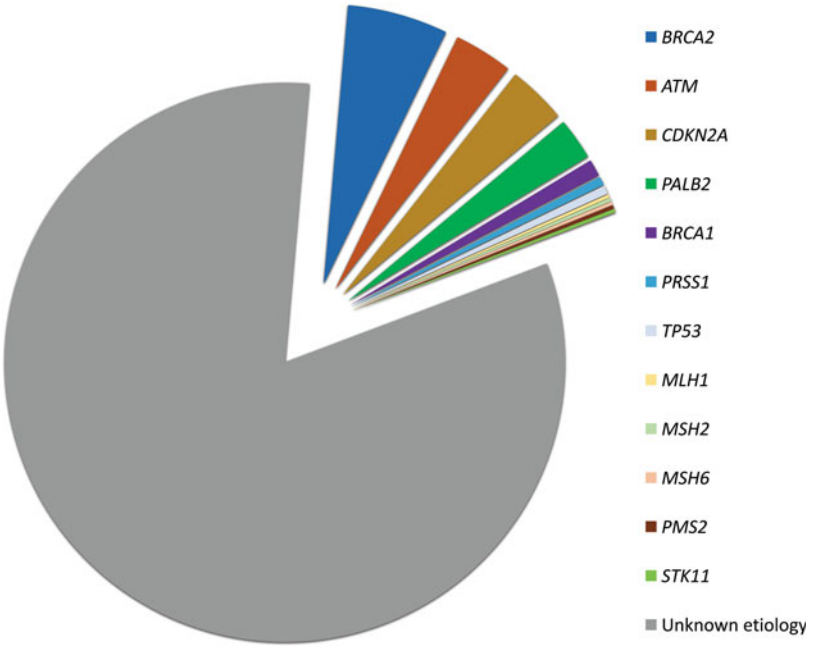

Fig 2 Fraction of familial pancreatic cancer attributable to established susceptibility genes

Familial Pancreatic Cancer Susceptibility Genes

Ataxia-Telangiectasia Mutated (ATM)

ATM is a 3,056-amino acid, 351 kDa, serine-threonine kinase that mediates DNA double-strand break repair through association with the MRE11–RAD50–NBS1 (MRN) complex, autophosphorylation of serine residues, and activation of numerous downstream effectors [32]. Located on chromosome 11, inheritance of biallelic deleterious germline variants in *ATM* results in the classic clinical syndrome ataxia-telangiectasia (A-T). A-T is a rare disorder that results in progressive neurological symptoms such as cerebellar ataxia, cutaneous telangiectasias, immunological deficiencies, and cancer susceptibility [32].

Using an unbiased approach to search for pancreatic cancer susceptibility genes, Roberts and colleagues sequenced the germline exomes of 22 individuals from 10 families and the germline genomes of 16 individuals from 6 families. Using a filter-based approach, they identified heterozygous deleterious germline variants in *ATM* in two families that segregated with disease. In a replication cohort of 166 familial pancreatic cancer patients and 190 spousal controls, four heterozygous deleterious germline variants were found in cases compared to none in controls. This association was even stronger for the most severely affected familial pancreatic

cancer kindreds, with three or more affected relatives [33]. This finding has been replicated in other studies [31, 34, 35]. Furthermore, whole-exome sequencing of pancreatic adenocarcinomas identified *ATM* somatic alterations, mutations, and copy number loss events, in 8% of patients, lending further support for the role that *ATM* plays in pancreatic tumorigenesis [36].

BRCA2, DNA Repair Associated (BRCA2)

BRCA2, also known as *FANCD*, encodes a protein whose function is to repair DNA double-strand breaks and interacts with *BRCA1* and *PALB2*. *BRCA2* was first identified through the study of families with an aggregation of early-onset breast cancer as well as the observation of a homozygous deletion in the region harboring the *BRCA2* gene on chromosome 13 in a pancreatic cancer. Women with a deleterious mutation in *BRCA2* have marked increase risk of cancer. In particular, they carry a 49% (95% CI, 40–57%) lifetime risk of breast cancer and an 18% (95% CI, 13–23%) risk of ovarian cancer. Males are at an increased risk of breast cancer as well. The first study identifying an important role for germline *BRCA2* mutations in pancreatic cancer risk was a case series of 41 pancreatic cancer patients where 4 (7%) harbored deleterious *BRCA2* mutations [37]. The prevalence of deleterious *BRCA2* mutations does increase as family history of pancreatic cancer increases with up to 16% of patients from families with three or more pancreatic cancers carrying germline *BRCA2* mutations [38]. A German study identified 12% of patients from familial pancreatic cancer kindreds had deleterious *BRCA2* mutations [39]. In 180 pancreatic cancer patients with either a first- or second-degree relative with pancreatic cancer, ten deleterious germline mutations in BRCA2 were found, representing 6% of familial kindreds [40]. While the prevalence of deleterious *BRCA2* mutations is higher among those with familial pancreatic cancer, a significant fraction of pancreatic cancer patients with apparently sporadic disease are also found to have deleterious *BRCA2* mutations. This was first demonstrated in an initial study by Goggins et al. and supported by more recent studies including a Canadian study where up to 3.6%, pancreatic cancer patients, unselected for family history, were found to have deleterious mutations in *BRCA2* [41]. Similarly, 4.6% of unselected Ashkenazi Jewish pancreatic cancer patients undergoing resection are reported to harbor a deleterious germline mutation in BRCA2 [42]. Given the strong association of *BRCA2* mutations with risk of breast and ovarian cancers, many pancreatic cancer patients with deleterious *BRCA2* mutations report a family history of one of these cancers. However many do not [37, 38].

There is still considerable uncertainty of the precise risk of pancreatic cancer associated with *BRCA2* mutations, in part because the studies of lifetime risk of pancreatic cancer among *BRCA2* carriers are limited to families ascertained based on history of breast/ovarian cancer. These studies suggest the *BRCA2* mutation carriers have a 3.51–5.79-fold increased risk [43, 44] of pancreatic cancer.

BRCA1, DNA Repair Associated (BRCA1)

Like BRCA2, the BRCA1 gene plays an important role in DNA repair [27, 28] and confers an increased risk of pancreatic cancer. Mutations in *BRCA1* confer a lifetime risk of breast cancer of 57% (95% CI, 47–66%) and lifetime risk of ovarian cancer of 40% (95% CI, 35–46%). Studies examining the association of *BRCA1* and pancreatic cancer are less consistent than those examining the association between *BRCA2* and pancreatic cancer. One study reported a *BRCA1* mutation prevalence of 1.2% among familial pancreatic cancer patients [45]. However, other studies did not report an excess *BRCA1* mutations among patient with pancreatic cancer [42, 46], but this lack of association could be due to a lack of power to detect the modest association between *BRCA1* and pancreatic cancer. This risk of pancreatic cancer among BRCA1 carriers, as ascertained from kindreds with a clustering of breast and ovarian cancer, is 2.26–4.11 fold higher than the general population [44, 47]. A family history of breast and ovarian cancer in addition to pancreatic cancer can strongly suggest a *BRCA1* mutation. This is particularly true for ovarian cancer where a significant fraction of ovarian cancer is explained by *BRCA1* mutations. However, not all pancreatic cancer patients with inherited *BRCA1* mutations present with a family history of pancreatic, breast, or ovarian cancer.

Partner and Localizer of BRCA2 (PALB2)

PALB2, also known as *FANCN*, encodes a protein that is a critical effector in homology-directed repair of DNA double-strand breaks and interacts with *BRCA1* and *BRCA2* [48, 49]. PALB2 is also a component of the Fanconi anemia pathway, and biallelic deleterious germline variants result in a Fanconi anemia phenotype similar to loss of *BRCA2* [49]. Furthermore, monoallelic deleterious germline variants result in an increased risk of breast and pancreatic cancers.

The identification of *PALB2* mutations in individuals with a family history of pancreatic cancer was the first study to demonstrate that whole-exome sequencing can identify the cause of a hereditary disease. In this study, the entire coding regions of 20,661 genes were sequenced in germline and tumor DNA from a patient with familial pancreatic cancer [50]. Jones and colleagues employed a novel filter-based approach and were able to identify a germline heterozygous, protein-truncating variant in *PALB2* that was rare in the general population, and importantly, occurring with a somatic mutation in *PALB2* in the tumor of the sequenced patient. In an independent panel of 96 familial pancreatic cancer patients, the authors identified three patients with premature truncating variants in *PALB2*. These observations provided the first evidence implicating *PALB2* in pancreatic cancer susceptibility, and it was the first time the gene responsible for an inherited syndrome had been identified using whole-exome sequencing.

Since the initial identification of *PALB2* as a familial pancreatic cancer susceptibility gene, additional studies have confirmed the association of deleterious germline

variants in *PABL2* with pancreatic cancer. In one study, *PALB2* was sequenced in 254 patients with either sporadic or familial pancreatic cancer resulting in the identification of a single patient with a heterozygous germline deletion that encompassed multiple exons [51]). In a European study of 81 familial pancreatic cancer families without a known deleterious germline variant in *BRCA2*, three deleterious variants in *PALB2* were identified [52]. In a recent Pancreatic Cancer Genetic Epidemiology (PACGENE) Consortium study, 727 unrelated pancreatic cancer patients, including 521 patients that met the criteria for familial pancreatic cancer, had their germline DNA sequenced to Clinical Laboratory Improvement Amendments (CLIA) standards. In this study, only four deleterious germline variants, representing 0.6% of sequenced patients, were identified in *PALB2*. Similarly, Roberts and colleagues sequenced the germline genomes of 638 familial pancreatic cancer patients from 593 kindreds and found 5 deleterious variants in PALB2, representing 0.8% of kindreds [31].

The lifetime risk of pancreatic cancer in individuals with a deleterious germline variant in *PALB2* is still unclear. Relatives of breast cancer patients with a deleterious germline variant in *PALB2*, however, have a 5.93-fold increased risk of developing breast cancer (95% confidence interval, 2.41–14.56) [53]. Recent evidence, however, suggests that deleterious germline variants in *PALB2* explain about 1% of familial pancreatic cancer. Future studies will be needed to assess the magnitude of increased risk associated with deleterious germline variants of *PALB2* and assess whether routine clinical testing of familial pancreatic cancer patients is warranted.

Mismatch Repair Genes (MLH1, MSH2, MSH6, PMS2)

The mismatch repair genes *MLH1*, *MSH2*, *MSH6*, and *PMS2* are essential components of DNA repair resulting from base pair mismatches during replication. Deleterious germline variants in these genes result in Lynch syndrome (also known as hereditary nonpolyposis colorectal cancer), an autosomal dominant condition that carries a significant lifetime risk of colorectal cancer. Tumors arising in Lynch syndrome patients are deficient in mismatch repair due to the presence of a deleterious germline variant and second somatic hit in the same mismatch repair gene. As a consequence, tumors in Lynch syndrome patients have demonstrable microsatellite instability (MSI) and a high number of somatic mutations.

In addition to colorectal cancer, patients with Lynch syndrome are also at an increased risk of extracolonic malignancies that include urinary tract cancers, endometrial cancer, breast cancer, small intestinal cancer, gastric cancer, liver cancer, prostate cancer, and pancreatic cancer [54]. The increased risk of pancreatic cancer associated with a deleterious germline variant in a mismatch repair gene is estimated to be 8.7-fold to age 70 (95% confidence interval: 4.7–15.7-fold) [55]. Recent genome-wide sequencing studies have found limited numbers of such deleterious germline variants in familial pancreatic cancer patients, with estimated prevalence between 0% and 2.1% [35], [31]. Lynch syndrome, therefore, may be a rare cause of familial pancreatic cancer.

Protease Serine 1 (PRSS1)

The *PRSS1* gene encodes cationic trypsin. Inherited inactivating mutations in this gene result in hereditary pancreatitis, a debilitating disorder of early-onset, recurrent, severe pancreatitis that affects one to six people per million [56]. While deleterious germline variants in *PRSS1*, specifically p.R122H and p.N29I, are most commonly observed in hereditary pancreatitis patients, other genes are also known to contribute either directly or indirectly through multigene interactions to increase pancreatitis risk; these include variants in *SPINK1*, *CPA1*, *CTRC*, and *CFTR* [56, 57].

Hereditary pancreatitis patients also have a significantly increased risk of pancreatic cancer, with a cumulative risk to age 70 of 40–44% and age 75 of 54% [57–59]. Hereditary pancreatitis patients who are smokers are twice as likely to develop pancreatic cancer, and the mean age of onset of pancreatic cancer in smokers with chronic pancreatitis is 20 years younger than hereditary pancreatitis patients who are not smokers [60]. Interestingly, compared to all other established familial pancreatic cancer susceptibility genes, *PRSS1* is not a tumor suppressor gene, and its action is not intrinsic to the pancreatic cancer cell of origin. Instead, *PRSS1* acts an external factor to promote tumorigenesis, presumably through repeated instances of injury, inflammation, and repair.

Serine/Threonine Kinase 11 (STK11)

STK11, also known as liver kinase B1 (LKB1), is a serine/threonine kinase located on chromosome 19. Inherited deleterious variants in *STK11* are the predominant cause of Peutz–Jeghers syndrome, an autosomal dominant condition associated with hamartomatous polyps of the gastrointestinal tract and mucocutaneous hyperpigmentation [61]. Patients with Peutz–Jeghers syndrome also have a greatly increased risk of various gastrointestinal and extra-gastrointestinal malignancies [62, 63]. Specifically, in a large series of Italian Peutz–Jeghers syndrome patients, relative overall cancer risk was 15.1-fold higher than the general population, with gastrointestinal and pancreatic malignancies showing the greatest increases of 126.2-fold and 139.7-fold, respectively [64].

Cyclin-Dependent Kinase Inhibitor 2A (CDKN2A)

CDKN2A is a tumor suppressor gene that encodes both the $p16^{INK4A}$ and $p14^{ARF}$ proteins through transcription of alternate open reading frames. p16 acts to limit cell cycle progression through interactions with cyclin-dependent kinase 4 (CDK4) that inhibits retinoblastoma protein phosphorylation and subsequent release of E2F transcription factors [65]. p14 expression results in p53 stabilization and inhibition of cell cycle progression through binding MDM2, a negative regulator of p53 [66]. As a critical component of several cell cycle pathways, it is

unsurprising that *CDKN2A* is the most commonly mutated or deleted tumor suppressor gene in pancreatic cancers, with functional loss in up to 90% of tumors [67]. Furthermore, somatic mutation or deletion of *CDKN2A* appears to be an early event in pancreatic tumorigenesis, with functional loss observed in pancreatic intraepithelial neoplasms, an early pancreatic adenocarcinoma precursor lesion [68–70].

Deleterious germline variants in *CDKN2A* are the underlying cause of melanoma in up to 40% of families with an inherited predisposition to the disease [71, 72]. Deleterious germline variants in *CDKN2A* have been also identified in individuals with pancreatic cancer. McWilliams and colleagues found that 0.6% of pancreatic cancer patients unselected for family history had deleterious germline variants in *CDKN2A* [73]. When considering only those patients with a family history of pancreatic cancer, 3.3% of patients had a deleterious variant in *CDKN2A*.

The risk of pancreatic cancer in individuals harboring a deleterious germline variant in *CDKN2A* is increased 32-fold (95% confidence interval: 1.5–47.7-fold), with an estimated cumulative risk of 57.6% by age 80 (95% confidence interval, 7.8–85.7%) (74). The risk of pancreatic cancer is also significantly increased 7.4-fold (95% confidence interval: 2.3–18.7-fold) in first-degree relatives of melanoma patients with a deleterious germline variant in *CDKN2A* compared to the first-degree relatives of melanoma patients without a deleterious germline variant in *CDKN2A* [74]).

Candidate Familial Pancreatic Cancer Susceptibility Genes

Advances in sequencing and genotyping technology over the last 10 years have allowed rapid, high-throughput genome-wide determination of germline single nucleotide variants, insertions, deletions, and copy number alterations in individuals with familial pancreatic cancer. These powerful technologies have been coupled with filter-based analyses that utilize operator-defined criteria and thresholds integrating variant-level, gene-level, and population-level data with knowledge of disease epidemiology and genetics. Such approaches have led to the identification of the familial pancreatic cancer susceptibility genes *ATM* and *PALB2* [33, 50].

Recent whole-genome and whole-exome sequencing of familial pancreatic cancer patients has highlighted the genetic heterogeneity underlying susceptibility to pancreatic cancer and the difficulties in identifying further susceptibility genes [31]. Using a filter-based approach to assess variants most likely deleterious to and contributing to pancreatic cancer susceptibility, specifically, rare heterozygous premature truncating variants, has led to the identification of candidate susceptibility genes, for example, *APC*, *BUB1B*, *CPA1*, *FANCC*, *FANCG*, *FAN1*, *NEK1*, and *RHNO1* [31, 75]. Interestingly, several of these candidate genes are associated with other hereditary cancer syndromes, implicated in DNA repair, or chromosome maintenance. Furthermore, deleterious germline variants in *CPA1* have recently been

associated with hereditary pancreatitis, a significant risk factor for pancreatic cancer [76, 77]. However, further validation and characterization of these candidate genes is necessary before integrating them into clinical decision-making.

Low-Risk Common Genetic Variants Associated with Pancreatic Cancer

The development of high-density SNP arrays enabled large-scale genome-wide association studies to identify low-penetrance common genetic variants that are associated with pancreatic cancer risk. To date, genome-wide association studies have identified common variants in the following regions as significantly associated with pancreatic cancer risk: 9q34 (*ABO*), 13q21, 1q31 (NR5A2), 5p15.33 (*CLPTM1L* and *TERT*), 7q32.3, 16q23.1 (*BCAR1/CTRB1/CTRB2*), 13q12.12 (*PDX1*), 22q12.1 (*ZNRF3*), 2p13.3 (near *ETAA1*), 3q29 (*TP63*), 7p13 (*SUGCT*), and 17q25.1 (*LINC00673*) [78–82]. While each of these variants has only a small effect on pancreatic cancer risk, with per-allele odds ratios ranging from 1.1 to 1.3, overall they explain approximately 3% of the underlying heritability of pancreatic cancer. Many of these same variants have been shown also to have a similar association with familial pancreatic cancer [9].

Screening of High-Risk Individuals

The overall 5-year survival rate for pancreatic is less than 8%. Survival among individuals with early-stage disease who undergo surgical resection exceeds 40%. Identifying early-stage disease or individuals with advanced precursor lesions including high-grade intraductal papillary mucinous neoplasm (IPMN) or high-grade pancreatic intraepithelial neoplasia (PanIN-3) offers the best hope for potentially curative therapeutic interventions.

While early detection screening is not recommended, consensus screening guidelines have been developed to guide ongoing early detection studies for high-risk individuals. For screening studies, high-risk is typically defined as a first-degree relative of a patient meeting the criteria for familial pancreatic cancer, an individual with a known deleterious germline variant in a familial pancreatic cancer susceptibility gene, and at least one affected first-degree relative [83]. Ideally, all screening should occur as part of an ongoing clinical trial or at a center with expertise in early detection screening for pancreatic cancer. Screening of these patients is recommended to include endoscopic ultrasound (EUS) and/or magnetic resonance imaging (MRI). While there is also considerable debate about when to begin screening, most studies begin screening in the fifth decade of life or 10 years younger than the earliest age-of-onset of pancreatic cancer in the family. As not all high-risk patients have the same pancreatic cancer risk, the diagnostic yield as measured by incident cases detected is likely to vary based on patient characteristics including germline mutation status.

Personalized Therapeutic Approaches

As cancer is in essence a genetic disease, much effort has been made to identify novel therapeutic approaches to target the specific genetic changes underlying the development of a tumor in a patient. Knowledge of the genetics underlying pancreatic cancer susceptibility in familial pancreatic cancer provides an uncommon opportunity to realize the promise of such personalized therapeutic approaches.

Biallelic loss of *ATM*, *BRCA1*, *BRCA2*, or *PALB2* in the tumor results in defects in DNA double-stand break repair and an opportunity for personalized therapeutic approaches. Biallelic loss of one of these genes in high-risk patients is often the result of a deleterious germline variant and a second somatic mutation or loss-of-heterozygosity event in the tumor. Biallelic somatic loss of one of these genes, however, is also a possibility and would result in similar therapeutic vulnerability. Specifically, patients with tumors harboring defects in homology-directed DNA double-strand break repair are more susceptible to DNA-damaging agents such as platinum-based chemotherapy, DNA cross-linkers including mitomycin-C [84], and ionizing radiation. Furthermore, such tumors are also susceptible to poly [ADP-ribose] polymerase 1 (PARP-1) inhibitors through synthetic lethal inhibition of base excision repair [85–90].

Another subset of patients that may benefit from personalized therapeutic approaches are those with mismatch repair-deficient tumors. Similar to defects in DNA double-strand break repair, mismatch repair-deficient tumors can occur either through the acquisition of an inherited deleterious germline variant in one of the four mismatch repair genes (*MLH1*, *MSH2*, *MSH6*, *PMS2*), coupled with a somatic alteration in the same gene, or purely by virtue of somatic loss of one of these genes. In either situation, mismatch repair-deficient tumors are more susceptible to programmed death 1 (PD-1) blockade than tumors proficient in mismatch repair [91]. While reported inheritance of deleterious variants in mismatch repair genes and mismatch repair deficiency in pancreatic cancers is uncommon and possibly associated with a medullary phenotype [92], the responses seen in this subset of patients warrant appropriate germline and/or tumor analysis and classification [31, 35].

Conclusion

The understanding of the genetic etiology of pancreatic cancer in high-risk individuals remains incomplete. Despite recent advances in the understanding of the genetic basis of pancreatic cancer risk, the etiology of increased risk in the majority of familial pancreatic cancer kindreds is still unknown and only a fraction of the heritability of pancreatic cancer is explained. In addition, improved early detection methods are needed in order to reduce the burden of pancreatic cancer in these high-risk populations. Finally, knowledge of the inherited and somatic genetics that underlie the development of pancreatic cancer has led to advancements in personalized therapies, for example, the use of PARP-1 inhibitors in patients with

homology-directed repair-deficient tumors or the use of PD-1 inhibitors in patients with mismatch repair-deficient tumors. Future efforts are necessary to guide patient selection, assess combination therapies, and determine optimal dosing strategies to fully leverage these therapies in the treatment of pancreatic cancer.

Cross-References

- ▶ Epidemiology and Prospects for Prevention of Pancreatic Cancer
- ▶ Inherited Pancreatic Endocrine Tumors
- ▶ Management of Cystic Neoplasms of the Pancreas Including IPMNs
- ▶ Precision Medicine Based on Next-Generation Sequencing and Master Controllers
- ▶ Secondary Screening for Inherited Pancreatic Ductal Adenocarcinoma

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Inherited Pancreatic Endocrine Tumors

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Abstract

Pancreatic neuroendocrine neoplasias (pNENs) may arise sporadically or in the setting of an inherited tumor syndrome. These syndromes comprise the multiple endocrine neoplasia type 1 (MEN1), the von Hippel-Lindau (VHL) syndrome, and the neurofibromatosis type 1 (NF-1). The prevalence and the different entities of pNENs differ significantly between these syndromes resulting in distinct treatment and screening recommendations.

Treatment of pNENs in the setting of an inherited tumor syndrome should consider the natural history of the disease, clinical symptoms, and the potential for malignant transformation which has to be considered individually for every patient.

Keywords

Pancreatic neuroendocrine neoplasia · Multiple endocrine neoplasia type 1 · Neurofibromatosis type 1 · Von Hippel-Lindau syndrome · Screening · Practice guidelines

Multiple Endocrine Neoplasia Type 1 (MEN1)

Introduction

MEN1 is an autosomal dominant inherited disease caused by germ line mutations in the *Menin* gene on chromosome 11q13 [1–3]. It has a penetrance of over 90% by the age of 50 years, and the incidence is estimated to be between 2 and 20 per

100 000 [4]. As first described by Wermer in 1954, affected patients display an "adenomatosis of endocrine glands" [5]. Before 1997, when the *Menin* gene was identified, an involvement of more than two characteristically affected organs was suspicious for MEN1. Patients can develop endocrine lesions in the parathyroid glands, the pancreas or duodenum, the anterior pituitary gland, and the adrenals, respectively. The wide spectrum of tumors also includes neuroendocrine tumors of thymus and bronchial tree, lipomas, cutaneous fibromas, and thyroid neoplasms (Table 1). Since the identification of the causative *Menin* gene, more than 1,000 mutations have been identified [3]. So far genotype-phenotype studies have not detected any statistical relevant direct correlations [6]; nevertheless, in some family cases, recurrent tumor patterns are notified. The large MEN1 cohort of the GTE (Groupe d'etude des Tumeurs Endocrines) group revealed that MEN1 patients harboring a mutation in the JunD interaction domain have a higher risk of death [7]. A retrospective analysis of a prospective collected database revealed that there exists a genotype-phenotype correlation regarding pNENs. MEN1 patients with mutations leading to CHES1-LOI (loss of interaction with the checkpoint kinase 1) have a higher risk of malignant pNENs with an aggressive course of disease. Furthermore, an aggressive course of disease was hypothesized for MEN1 patients with large *MEN1* gene deletions.

Table 1 Expression of MEN1

Affected organ	Tumor	Frequency (%)	Hormone	Clinical syndrome
Parathyroid gland	Hyperplastic parathyroid	90	Parathyroid hormone	Primary hyperthyroidism
Pancreas and duodenum	Gastrinoma	20–30	Gastrin	ZES
	Insulinoma	5–10	Insulin	Hypoglycemia
	NF-pNEN	50–80	PP	None, local tumor growth
	Vipoma	1	VIP	WDH
	Glucagonoma	3	Glucagon	Glucagonoma S
Pituitary gland	Prolactinoma	20–60	Prolactin	Galactorrhea
	nf		None	Visual loss
Adrenal gland	nf	20–60	None	None
	f		Aldosterone, cortisol	Cushing's S, Conn S
Thymus	NEN	2	CgA	
Lung	NEN	3	Serotonin, CgA	Carcinoid S
Stomach	NEN	3	CgA	
Skin	Lipoma	Up to 20	None	None
	Fibroma	Up to 80	None	None

ZES Zollinger-Ellison syndrome, *nf-pNEN* nonfunctioning pancreatic neuroendocrine neoplasia, *PP* pancreatic polypeptide, *VIP* vasoactive intestinal polypeptide, *WDH* watery diarrhea and hypokalemia, *nf* nonfunctioning, *NEN* neuroendocrine neoplasia, *CgA* chromogranin A, *S* syndrome

Clinical symptoms which are associated with hormone excess comprise in declining frequency hypercalcemia, nephrolithiasis, peptic ulcer disease, hypoglycemia, visual field loss, galactorrhea-amenorrhea, and rarely Cushing's syndrome. The onset of the different manifestations varies considerably, although hypercalcemia is frequently the first manifestation by the age of 20, followed by Zollinger-Ellison syndrome between 30 and 40 years of age.

Primary hyperparathyroidism is observed in up to 97% of MEN1 patients, and the parathyroid glands are therefore the most frequently affected organs [1]. Pancreatic neuroendocrine neoplasias (pNENs) are the second most manifestation with a frequency of 60–90%. Since medical treatment of ulcer disease has improved by introducing proton pump inhibitors (PPI), malignant pNENs became the most important determinant of survival in MEN1 patients [8]. PNENs can be either functioning (gastrinoma, insulinoma, vipoma, glucagonoma) or nonfunctioning. Gastrinomas, which are mostly located in the duodenal wall, account for 60% of functioning pNENs followed by insulinoma with approximately 20%.

Patients with MEN1 have a decreased life expectancy, with a 50% probability of death by the age 50. The major determinant of survival is malignant pNENs (G1/G2), including malignant gastrinomas, since up to 50% develop liver or other distant metastases [8]. The surgical management of pNENs in MEN1 patients remains controversial, because they have unique features compared to sporadic pNENs. They are multiple and distributed through the entire pancreas, which has been proven in autopsy studies and studies with resected specimen of MEN1 patients [9]. However, total pancreatectomy seems to be an "overtreatment" in these patients, especially since postoperative brittle diabetes might be a life-threatening condition.

Lifelong screening comprising careful hormonal assessments and regular imaging studies is supposed to detect malignant transformation at the earliest stage and is therefore strongly emphasized in current expert clinical practice guidelines for MEN1 patients. In addition, if MEN1 is suspected based on the personal and family history, a genetic testing of the index patient for a *MEN1* gene mutation should be performed after genetic counseling. The identification of a *MEN1* mutation in the index patient gives the possibility of a predictive genetic testing of family members after obligate genetic counseling. Mutation-positive family members should be enrolled in controlled screening programs, whereas mutation-negative family members can be omitted from such screening.

Natural History of pNENs in Patients with MEN1

The natural history of pNENs in MEN1 patients is still difficult to define due to the variability and the rarity of the disease. Approximately 30–50% of MEN1-associated pNENs are functional and cause symptoms and distinct syndromes by a hypersecretion of distinct hormones (e.g., gastrin, insulin). Nonfunctioning pNENs (NF-pNENs) are responsible for the other 50–70% of pNENs and are characterized by the absence of peptide hypersecretion (a part from pancreatic polypeptide (PP)). They

sometimes become symptomatic due to local tumor growth and/or advanced disease and are commonly detected during regular screening. PNENs in MEN1 patients are often multiple (up to 50), and NF-pNENs often coexist besides a clinically dominant functioning lesion. Since 80% of MEN1 patients develop pNENs and these tumors represent the most common disease-related cause of death, the identification and management of these lesions requires high awareness.

Gastrinoma is the most common functional pNEN in MEN1 patients and in contrast to its sporadic counterpart located in over 90% within the duodenal wall underlying the mucosa [10]. Duodenal tumors are often small measuring from 1 to 10 mm and had developed lymph node metastases in 40–60% at the time of diagnosis [11, 12]. However distant metastases to the liver and bones are less frequent than in sporadic disease, and MEN1-associated gastrinoma is suggested to follow a less aggressive course compared to its sporadic counterpart [13]. Nevertheless Gibril et al. report also an aggressive gastrinoma phenotype in 23% of MEN1 patients which is associated with large (>30 mm) pancreatic tumors, high serum gastrin levels, and liver and bone metastases [14].

Insulinoma is the second most frequent functioning pNEN in MEN1 patients with a prevalence of 10–20% [15]. Malignancy has been rarely reported and may develop in up to 9% of patients [16]. Coexistence with gastrinoma is observed in approximately 10%, although one tumor is dominating the hormone excess and consequently the clinical syndrome.

Vipomas occur rarely, are almost exclusively malignant, and are located in the pancreatic body or tail. Patients suffer from watery diarrhea with severe electrolyte disbalances, especially if they present already with liver metastases.

Glucagonomas develop in less than 3% of MEN1 patients and glucagon excess is not necessarily associated with a clinical syndrome. Especially small tumors (<3 cm) are often asymptomatic, but tumors are usually large and tend to be malignant in up to 80% [17, 18]. In cases with diffuse metastases, migratory, necrolytic skin rash, glossitis, stomatitis, angular cheilitis, diabetes, severe weight loss, and diarrhea may occur (Table 1).

Nonfunctioning pNENs with a prevalence of 50–80% are increasingly diagnosed based on modern imaging modalities in controlled screening programs. A high prevalence of these lesions could already be detected in young MEN1 patients in the second decade of life [19]. The malignant potential of these tumors varies considerably, but the tumor size seems to be a predictor for malignant transformation. In small retrospective series, an incidence of 20% lymph node metastases (LNM) in tumors larger than 1 cm and an incidence of LM (liver metastases) of 30% in tumors larger than 2 cm have been reported, which means vice versa that LNM and LM have not been observed in tumors smaller than 1 cm [20, 21]. The increasing number of resected NF-pNENs in prospective controlled screening programs revealed that malignancy is rarely observed in tumors smaller than 10–20 mm. Follow-up studies with endoscopic ultrasound suggested that most small NF-pNENs grow very slowly, but they definitely own a malignant potential [22] (Table 2).

Table 2 Screening in MEN1 at the Marburg ENETS Center of Excellence

Screening in MEN1 patients	
Biochemical (annually)	
Parathyroid glands	Calcium, parathyroid hormone
pNEN	Gastrin, pancreatic polypeptide, chromogranin A
	Fasting test, if an insulinoma is suspected
	Secretin provocation test, if a gastrinoma is suspected
Pituitary gland	Prolactin, IGF-1, ACTH
Imaging	
MRI abdomen	Annually or if tumor is suspected
Ga-68 DOTATOC-PET/CT	If tumor is suspected or every 2–3 yrs.
MRI of the pituitary gland	Every 3 yrs. or in case of hormone excess, visual impairment
EUS	Annually or if tumor is suspected
CT of the chest	Every 3 yrs. or if a thymic or bronchial carcinoid is suspected

pNEN pancreatic neuroendocrine neoplasia, *IGF-1* insulin-like growth factor 1, *ACTH* adrenocorticotropic hormone, *5-HIAA* 5-hydroxyindoleacetic acid, *yrs.* years, *MRI* magnetic resonance imaging, *CT* computed tomography

Clinical Management

Regarding the surgical management of MEN1-associated pNENs, the diagnostic workup and the surgical strategy have to be adopted to the tumor entity, the patients' health condition, and his/her preferences after detailed counseling. However, some controversies exist concerning the extent, timing, and benefit of pancreatic resections in MEN1 patients, especially since profound evidence-based data are still lacking. However, a consensus conference has proposed guidelines for the treatment of MEN1-pNENs [1].

Gastrinoma

Clinical Symptoms

The clinical appearance of MEN1-associated Zollinger-Ellison syndrome is similar to its sporadic counterpart (see previous chapter). It is characterized by abdominal pain due to peptic ulcers and heartburn with or without diarrhea. Hypercalcemia increases symptoms in MEN1 patients with concomitant primary hyperparathyroidism. Quite the contrary is observed in patients after parathyroid surgery with hypocalcemia resulting in milder symptoms and even false-negative secretin provocation tests. This has led to the recommendation to first cure the pHPT before the resection of gastrinoma [23].

Diagnostic Procedures

The diagnosis is established by clinical symptoms, an elevated serum gastrin level in the presence of acid in the stomach (pH <4), and a positive secretin-provocation test

(see sporadic gastrinoma). To prevent a false-positive secretin test, a coexisting primary hyperparathyroidism should be treated before testing and a 48-h pause of proton pump inhibitor treatment prior to secretin-provocation test should be initiated.

After the biochemical diagnosis is established, further workup should include endoscopic ultrasound (EUS) supplemented by magnetic resonance imaging (MRI) and SRS PET-CT imaging (e.g., Ga-68-DOTATOC-PET/CT) to visualize pNENs and potential metastases. In contrast to sporadic gastrinoma, MEN1-associated gastrinomas are predominantly localized in the second and third portion of the duodenum (50% vs. >90%) and are in the majority less than 10 mm in size. Therefore, they often cannot be localized by preoperative imaging. Although an exact preoperative localization of MEN1 gastrinoma is often difficult, the gastrin source can be regionalized by a selective arterial secretin injection test (Imamura technique) [24]. This regionalization facilitates the decision for the adequate surgical procedure which might include a pylorus-preserving partial pancreaticoduodenectomy. For further therapy in MEN1-ZES patients, it should be considered that the majority of these patients have concomitant pNENs besides gastrinomas [25].

Treatment

The target organ of MEN1-ZES is the duodenum and rarely the pancreas. The management of ZES in MEN1 patients is controversial reaching from medical treatment with proton pump inhibitors alone to extensive pancreatic resections. This controversy has several reasons. On one hand, the course of disease is rather mild, and MEN1-ZES is considered by many experts as a surgically incurable disease. Therefore, recent expert guidelines suggest medical management using PPI for the majority of patients [1]. On the other hand, it has been shown that medically treated ZES patients developed liver metastases more frequently than surgical-managed patients (29% vs. 5%) [26]. Thus, there is some evidence that surgery may reduce the malignant spread of gastrinoma and increase survival. However, there is no consensus on the indication and the timing of surgery, since there is yet no proven parameter that indicates an aggressive course of disease, and long-term survival is excellent in the majority of patients. As long as this is the case, an imageable pNEN >2 cm, although most likely nonfunctioning, seems to be a good surrogate parameter to indicate surgery in order to prevent distant metastatic disease. However, the higher chance of cure when performing a partial pancreaticoduodenectomy (PPD) resection at the time of biochemical ZES evidence should be discussed with regard to benefits and risks. Although there is disagreement on the optimal surgical procedure, it is obvious that any operation for MEN1-ZES should include duodenotomy or even resection of the duodenum to provide a chance of cure. PPD resection results in the highest chance of long-term biochemical cure [27], but the excellent long-term survival after less-aggressive non-PPD resections and the potential increased postoperative mortality and long-term morbidity of PPD resections make its current role unclear. Although prospective controlled studies are warranted to clarify these issues, it is unlikely that such long-term studies will be

performed given the rarity of the disease and the necessity of long-term protocols. Therefore, MEN1 patients should be cared for by multidisciplinary teams comprising relevant specialists with experience in the diagnosis and treatment of neuroendocrine tumors. It would be a major goal to identify molecular or other parameters that indicate an aggressive course of MEN1-ZES to facilitate the decisions regarding the timing and type of surgery. At present, the indication and type of surgical procedure should be individualized according to preoperative findings, patient's history (e.g., age, preexisting insulin-dependent diabetes), and patient's preference.

Some experts recommend an aggressive surgical approach as soon as the biochemical diagnosis of ZES is established [25]. The goal of this philosophy is to prevent the development of liver metastases and to improve long-term survival, although biochemical long-term cure might not be achieved.

Surgery can be indicated in patients with MEN1-ZES when diffuse metastatic spread has been excluded by preoperative imaging and a coexisting pHPT has been cured before. At surgery a duodenotomy and excision of palpable tumors and enucleation of pancreatic head tumors and spleen-preserving distal pancreatectomy to the level of the portal vein with peripancreatic lymphadenectomy as recommended by Thompson et al. [28] were considered the standard procedure. The biochemical cure rate of this procedure is low and varies between 0% and 33%, but the development of liver metastases during long-term follow-up does not exceed 16% (Table 3). Therefore, some authors proposed a pylorus-preserving partial pancreaticoduodenectomy (PPPD) for MEN1-ZES [27]. The rationale is that MEN1 is a genetically determined disease and that the ZES will recur as long as the target organ duodenum exists. In addition, it has been shown that MEN1-associated gastrinomas are associated with hyperplastic gastrin cell lesions and very small gastrin-producing microtumors less than 500 μm in diameter [29] which cannot be removed by local excision since they are not palpable. Finally, 95% of MEN1 gastrinomas are located within the gastrinoma triangle and occur multiple in the duodenum [30]. PPPD has been evaluated in smaller case series and achieved biochemical cure rates from 77% to 100% [27] (Table 4). However, before PPPD can be suggested as a standard procedure in MEN1 patients with ZES, much more data need to be analyzed, especially the long-term side effects that have to be

Table 3 Results after surgical excision and/or non-PD resections of MEN1-associated gastrinoma (Modified from Bartsch and Albers [25])

Authors	Patients (n)	ST normal (%)	LM (%)
Thompson [31]	40	13 (33)	1 (2.5)
Norton [32]	48	2 (4)	3 (6)
McFarlane [33]	10	0 (0)	0 (0)
Mignon [34]	36	1 (3.5)	5 (13)
Lopez [27]	9	3 (33)	0 (0)
Dickson [35]	11	3 (27)	1 (9)
Total	154	22 (15)	10 (6.5)

ST secretin provocation test, *LM* liver metastases

Table 4 Results after PD resections of MEN1-associated gastrinoma (Modified from Bartsch and Albers [25])

Authors	Patients (n)	ST normal (%)	LM (%)
Stadil [36]	3	3 (100)	0 (0)
Tonelli [37]	13	10 (77)	1 (9)
Dickson [35]	3	3 (100)	0 (0)
Imamura [38]	3	1 (33)	0 (0)
Lopez [27]	13	12 (91)	0 (0)
Total	35	31 (89)	1 (33)

PD pancreaticoduodenectomy, *ST* secretin provocation test, *LM* liver metastases

carefully evaluated. Pancreatic-preserving duodenectomy might be another favorable alternative, but it is a technically demanding procedure, and the cure rates are lower after PPD, and the morbidity is high.

In recurrent or persistent MEN1-associated ZES, surgery has to be carefully indicated in every patient.

The decision depends on the severity of ZES, the type of the initial procedure, the presence of lymph node or liver metastases, and the patients' health condition. Given the relatively slow progression of the disease, the reoperation should avoid the situation of a total pancreaticoduodenectomy, since the side effects of this procedure might be more life-threatening than the ZES.

Prognosis

Compared to sporadic gastrinomas, MEN1-associated gastrinomas have a more favorable prognosis. The overall survival of operated MEN1-associated gastrinomas is excellent with 10- and 20-year survival rates of 96% and 85%, although 40–60% of patients have lymph node metastases at initial laparotomy [36].

Insulinoma

Clinical Symptoms

Symptoms are mainly caused by hypoglycemia and are described in detail in the chapter of sporadic pancreatic endocrine tumors.

Diagnostic Procedures

The biochemical diagnosis is established by a supervised positive 72-h fasting test, defined by a pathological insulin-glucose index and symptomatic hypoglycemia. CT, MRI, SRS imaging, and US demonstrated a decreased sensitivity (0–60%) in the

preoperative localization compared to EUS (60–95%) (see sporadic insulinomas). Most MEN1 patients have multiple, frequently nonfunctioning tumors in the pancreas making the identification of the insulinoma difficult. In some cases, with multiple pNENs >1 cm, it might be useful to perform a preoperative selective arterial calcium injection (SACI) angiography to regionalize the source of insulin overexpression.

Treatment

Like in sporadic insulinoma, surgery is always indicated, if the biochemical diagnosis of organic hyperinsulinism is established and diffuse metastatic disease is excluded by imaging. Surgical treatment options range from enucleation to partial pancreatectomy or distal pancreatectomy. Enucleation and limited resections are preferred as surgical treatment options and provide long-term cure for MEN1 patients with solitary dominant tumors [39], whereas a distal spleen-preserving pancreatectomy to the level of the portal vein with enucleation of pancreatic head tumors should be performed in patients with multiple, equally sized pNENs (Figs. 1 and 2). Nowadays the procedures can also

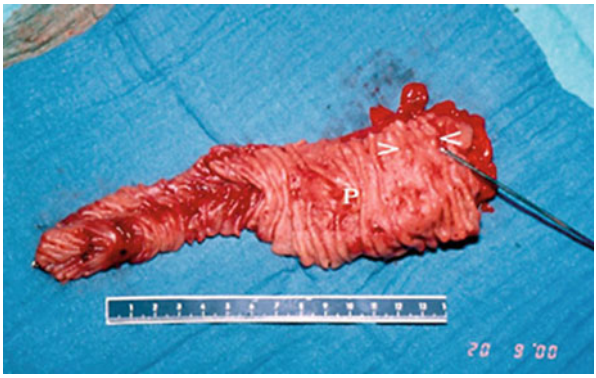

Fig. 1 Specimen after pylorus-preserving partial pancreaticoduodenectomy (PPPD) in a MEN1 patient with ZES (*arrows* indicate two small gastrinoma, *P* papilla vateri)

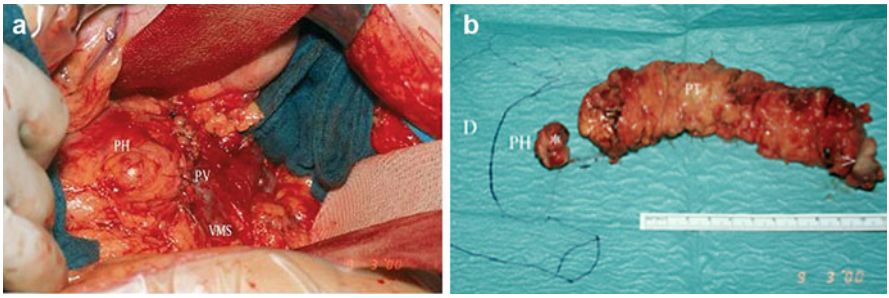

Fig. 2 Situs (**a**) and specimen (**b**) after distal pancreatic resection in a MEN1 patient with multiple NF-pNENs and insulinoma in the pancreatic head (*PH* pancreatic head insulinoma, *VMS* superior mesenteric vein, *PV* portal vein)

Table 5 Results of pancreatic surgery in MEN1-associated insulinoma

Authors	Surgery	PD/TP	DP	E	Cure	LM
Demeure [42]	6	0	5	1	84%	0
Grama [16]	7	0	NA	NA	57%	1
O'Riordain [43]	18	1	12	5	89%	0
Thompson [44]	7	0	7	0	100%	0
Lowney [45]	10	NA	NA	NA	NA	1
Bartsch [39]	13	1	4	8	92%	0
Laimore [21]	3	1	NA	NA	NA	NA
Baudin [46]	73	9	46	18	82%	0
Total	57	3	26	9	57–100%	2

PD pancreaticoduodenectomy, *TP* total pancreatectomy, *DP* distal pancreatic resection, *E* enucleation, *NA* not available, *LM* liver metastases

be performed safely using laparoscopic- and robot-assisted approaches [40, 41]. A peripancreatic lymphadenectomy is only mandatory, if malignancy is suspected by gross invasion or lymph node metastases.

Prognosis

Biochemical cure is achieved in 57–100% of cases in the absence of distant metastases (Table 5). MEN1 patients with an insulinoma are usually younger (20–30 years) than patients with sporadic insulinoma (40–60 years) [1]. Malignancy is rarely reported and occurs in 5–9%.

Vipomas and Glucagonomas

Vipomas and glucagonomas are rare functional pNENs in MEN1 patients occurring in 1–3% of patients. Malignancy is frequently observed occurring in 50–80% of patients [47].

Clinical Symptoms

Vipoma is associated with profuse watery diarrhea and hypotension, also referred to as WDHA syndrome. Tumors are often large (>5 cm) and liver metastases are frequently present at the time of diagnosis. Glucagon excess infrequently causes specific symptoms, but glucagonoma, usually large at diagnosis, may cause abdominal pain due to local tumor growth. In case of advanced tumors, a migratory, necrolytic skin rash might be the leading symptom. In addition, glossitis, stomatitis, angular cheilitis, diabetes, and severe weight loss may occur.

Diagnostic Procedures

The biochemical diagnosis is based on the measurement of elevated serum levels for VIP or glucagon. Preoperatively CT or MRI and SRS imaging should be performed to obtain an adequate staging.

Treatment

Recommendations for surgical treatment are rather based on general proposals following oncologic principles than on a widespread experience. The only chance of cure is the complete surgical resection, as these tumors are frequently malignant. Glucagonomas and vipomas are mainly located in the pancreatic body or tail making a distal splenopancreatectomy with peripancreatic lymph node dissection the procedure of choice. In case of pancreatic head vipoma or glucagonoma, a PPD should be performed. Debulking procedures are indicated if the majority (~90%) of the tumor burden can be resected, since they lead to an improvement of the clinical syndrome caused by the hormone excess.

Besides surgical approaches, medical treatment with somatostatin analogs (e.g., octreotide) or chemotherapy (e.g., streptozotocin and 5-fluorouracil or dimethyltriazeno-imidazole carboxamide) is also a successful options in some patients [1]. Target therapies as everolimus or sunitinib are novel therapy options which are recommended in patients with advanced and metastatic diseases [48]. In SSTR-positive tumors, peptide receptor radionuclide therapy (PRRT) is also a valuable option.

Nonfunctioning pNENs

The incidence of NF-pNENs in MEN1 patients varies from 30% to 80% [30, 50]. NF-pNENs in MEN1 patients have been reported to be malignant in 30–50% and are less frequently malignant than their sporadic counterparts with 70%. Retrospective data on sporadic NF-pNENs have revealed that 20% of patients with tumors larger >1 cm had lymph node metastases and 30% of patients with tumors >2 cm had liver metastases, respectively. However, there is no conclusive association between tumor size and risk of malignancy in MEN1-associated NF-pNENs. Even small (10–20 mm) NF-pNENs with lung and liver metastases have been reported in MEN1 patients [18]. In sporadic NF-pNENs, a lack of specific symptoms results in a delayed diagnosis associated with a poorer overall survival compared to functioning pNENs [51]. This is different in MEN1-associated NF-pNENs, since these tumors will be nowadays diagnosed early by regular screening due to the increased sensitivity of imaging methods. Thus, NF-pNENs are the most common tumors of the pancreaticoduodenal region in adult MEN1 patients. This is of importance as NF-pNENs are a significant cause of death in MEN1 mutation carriers [8, 52].

Clinical Symptoms

Symptoms are commonly unspecific, as hormone excess-related symptoms are lacking. In large tumors, local tumor growth-associated symptoms such as jaundice, abdominal pain or discomfort, and weight loss may frequently occur.

Diagnostic Procedures

After a careful biochemical evaluation in order to detect hormone oversecretion, especially with regard to subclinical ZES, imaging should include especially EUS and MRI of the abdomen. EUS is the superior preoperative imaging modality in MEN1 patients, especially if the tumor size is below 10 mm [53, 54]. It has to be highlighted that NF-pNENs in MEN1 are often multiple and may be associated with functioning tumors.

Treatment

The timing and extent of surgery are an ongoing discussion. In the past, some authors advocated the most aggressive approach with surgical exploration in case of biochemical evidence, even if imaging failed to visualize pancreatic lesions [49, 55]. The majority of authors indicated surgery when pNENs >10 mm in size could be visualized on imaging [56]. Meanwhile two retrospective studies have demonstrated that a surgical treatment is not beneficial [57, 58]. Therefore, current ENETS Consensus Guidelines recommend surgical resection only for tumors ≥2 cm [70]. In case of surgery, spleen-preserving distal pancreatectomy with enucleation of pancreatic head tumor or parenchyma-sparing enucleations of solitary pNENs are the preferred surgery procedures. Nowadays the procedures can be safely performed using minimal-invasive approaches.

Treatment of Liver Metastases in MEN1-Associated pNENs

Liver and other distant metastases are the most important predictor of survival in patients with MEN1 pNENs. The treatment in MEN1 patients with advanced disease attempts to reduce symptoms related to the hormone excess and to repress the tumor progression. Treatment options for metastatic MEN1-associated pNENs are the same as for sporadic pNENs, which are summarized in detail in the chapter of sporadic pancreatic endocrine tumors. If possible, cytoreductive surgery should be performed, even if a multivisceral resection is necessary. Other treatment options comprise biotherapy with somatostatin analogs and interferon, chemotherapy (streptozotocin, doxorubicin), targeted therapies (e.g., everolimus, sunitinib), embolization and chemoembolization, radiofrequency ablation, laser-induced tumor ablation, liver transplantation, peptide receptor radiotherapy, and selective intraarterial

radiotherapy [48]. In patients with ZES and metastatic, non-resectable distant metastases, symptoms can be controlled by high dose administration of proton pump inhibitors.

Screening and Surveillance in MEN1 Patients

Genetic testing for a *MEN1* mutation is suggested in patients suspicious for MEN1. The identification of a *MEN1* mutation in the index patient gives the possibility of a predictive genetic testing of family members. A predictive genetic testing requires obligating a genetic counseling prior testing. Mutation-positive family members should be enrolled in controlled screening programs according to the clinical practice guidelines for MEN1 patients [1], whereas mutation-negative family members can be spared from further investigations. However, in approximately 10% of patients with MEN1, a mutation cannot be identified. In these cases, large deletions of the *MEN1* gene should be tested.

Regular screening should include biochemical parameters and imaging procedures every 1–3 years according to the clinical practice guidelines for MEN1 [1] (see Table 2). Hormonal assessment should include PP, gastrin and CgA, calcium, intact parathyroid hormone, and secretin stimulation test (ZES). To avoid repeated radiation exposure, MRI is the preferred initial diagnostic tool to identify lesions in the pancreas, adrenal glands, lymph nodes, and liver. However, its accuracy in detecting small pNENs is limited, as duodenal tumors will always and pNENs smaller than 10 mm will often missed. SRS imaging, especially Ga-68 DOTATOC-PET/CT and EUS, is superior in the detection of pNENs in MEN1 patients (Table 2). Regular screening intends to detect lesions in involved glands at their earliest stage, especially to prevent the development of advanced metastatic disease by timely interventions.

Guidelines for screening in MEN1 patients, especially for pNENs, are provided by the NIH Consensus Conference 2012 [1] and the ENETS [60].

Von Hippel-Lindau Syndrome (VHL)

Introduction

The VHL syndrome is an autosomal dominant inherited syndrome that most commonly causes retinal, spinal, adrenal, renal, and pancreatic lesions. The annual incidence is estimated to be 1 of 36,000 with more than 90% penetrance by the age of 65 years [61]. The VHL gene, located at chromosome 3p25–26, is coding a tumor suppressor gene which plays a pivotal role in the transduction of hypoxia-driven signals. Over 200 mutations have been reported to be associated with the VHL syndrome, and the mutated VHL protein leads to an

Table 6 Phenotypes of VHL

Phenotype classification in families with VHL	
Type	Phenotype
Type 1	Retinal hemangioblastoma
	CNS hemangioblastoma
	Renal cell carcinoma
	Pancreatic neoplasms and cysts
Type 2A	Pheochromocytomas
	Retinal hemangioblastomas
	CNS hemangioblastomas
Type 2B	Pheochromocytomas
	Retinal hemangioblastomas
	CNS hemangioblastomas
	Renal cell carcinoma
	Pancreatic neoplasm and cysts
Type 2C	Pheochromocytomas

CNS central nervous system

increased transcription of hypoxia-induced genes. This results in an increased growth and survival of endothelial and stromal cells and lastly promotes their malignant transformation.

Regarding morbidity, the most serious lesions are hemangioblastomas and retinal angiomas as they impair the vision and other neurological functions. Mortality is mostly determined by renal cell carcinoma and malignant pNENs. VHL has been classified in four distinct phenotypes by the National Cancer Institute (Table 6) which represent the four clinical phenotypes 1, 2A, 2B, and 2C based on the different lesions [62]. Pancreatic neoplasms only occur in phenotypes 1 and 2B.

Prognosis

The lifetime expectancy in VHL patients was less than 50 years before surveillance protocols were developed. The major cause of death is renal cell carcinoma. Pancreatic lesions occur in 50–77% of VHL patients, most commonly pancreatic cysts and cystadenomas. The development of distant metastases of these both types of lesions has not been reported. Pancreatic neuroendocrine tumors (pNENs) are less common (9%) [63] but own a malignant potential. Pancreatic cysts or serous cystadenoma may coexist, but pNENs are usually smaller and solid. The median age of diagnosis is approximately 36 years, and the vast majority of pNENs are nonfunctioning. The most frequent sites of metastases are the liver and bones. Libutti reported that 17% of VHL patients with pNENs had distant metastases or developed them during follow-up [64]. The probability for malignancy increases with a tumor size of more than 30 mm from 0% to 20%.

Prevalence of Pancreatic Lesions and Clinical Symptoms

The most common pancreatic lesions are pancreatic cysts, which are present in 17–56% of VHL patients [65]. These lesions exhibit no malignant potential. Pancreatic cysts are detected commonly by CT or MRI scans of the abdomen or EUS by routine imaging in asymptomatic patients. Pancreatic cysts can rarely lead to duodenal compression and/or abdominal discomfort.

Serous cystadenoma is uncommon but has been reported to be associated with VHL. Lesions typically grow slowly, and malignant transformation in the setting of VHL has yet not been reported. Serous cystadenomas may lead to endocrine or exocrine insufficiency as well as to stenosis of the bile duct, if they grow to substantial size by compressing the pancreatic parenchyma.

Since almost all pNENs in VHL are nonfunctioning, they are clinically inapparent and will be generally detected during screening. Fifty percent of pNENs in VHL patients are located in the pancreatic head, whereas 25% each are located in the pancreatic corpus and tail, respectively.

Diagnostic Procedures

A hormonal assessment is not necessary since pNENs in VHL patients are usually nonfunctional. Imaging can be managed by CT scan or MRI and EUS which is superior in the detection of pNENs. A pheochromocytoma has to be excluded before scheduling VHL patients for pancreatic surgery.

Treatment

If cystadenoma or pancreatic cysts are symptomatic due to a compression of the bile duct, the duodenum pancreatic resection may be necessary.

There are no evidence-based guidelines with respect to the time point and the extent of surgery for pNENs in VHL patients. It has been suggested that the probability for malignancy increases significantly, if the tumor size exceeds 30 mm compared to tumors which are less than 3 cm [66]. Lesions between 10 and 30 mm require a personally adopted approach with respect to patients' age, comorbidity, and growth behavior. The extremely rare functioning pNENs and nonfunctioning pNENs exceeding 30 mm require surgical resection. Based on small series, most experts recommend follow-up by MRI or EUS every 12 months for lesions smaller than 30 mm [67]. The surgical strategy should aim to preserve as much pancreatic parenchyma as possible. Therefore, most experts recommend enucleation whenever feasible. Intraoperative ultrasound is obligatory to visualize the relationship of the tumor to the main pancreatic duct and major vessels. A laparoscopic approach is justified in preoperatively imaged lesions, if they are located in the pancreatic body/tail or in the ventral surface of the pancreatic head.

Screening and Surveillance

Although recent studies contributed to the understanding of phenotype-genotype correlation, mutation-based screening has yet not been recommended. Most experts warrant routine screening including all VHL-associated lesions. With respect to the endocrine manifestations, screening for pheochromocytoma in VHL type 2 patients comprises an assessment of catecholamine excretion in 24-h urine, MRI, and MIBG scan annually starting by the age of 10. Nonfunctioning pNENs should be screened by MRI or EUS every 1–2 years starting >16 years (www.vhl.org).

Neurofibromatosis (NF) Type 1

Introduction

Neurofibromatosis comprises a group of hereditary conditions predisposing to neurocutaneous manifestations. The genetically most amenable conditions are neurofibromatosis types 1 and 2. Neurofibromatosis type 1 is associated with pheochromocytoma, pNENs, and other tumor manifestations affecting the central and peripheral nervous system. Neurofibromatosis type 2 is characterized by bilateral acoustic neurinomas, whereas pheochromocytomas and pNENs are not part of this syndrome.

Neurofibromatosis type 1 affects 1 in 3,000 live births and 50% are caused by spontaneous mutations. The penetrance is almost 100%, but the clinical phenotype varies considerably. A phenotype-genotype correlation has not been defined so far. The *NF-1* gene is located on chromosome 17q11.2 and is coding for *neurofibromin* gene which acts as a tumor suppressor gene. *Neurofibromin* appears to be involved in the activation of the proto-oncogene *p21-Ras* and belongs to the family of Ras GTPases. Mutations of *neurofibromin* can result in a loss of inactivation of *p21-Ras*; in other words, the oncogene becomes activated.

The NIH criteria for neurofibromatosis lead to a safe diagnosis, if two or more of the following criteria are present [68]:

- Six or more café-au-lait macules over 5 mm in greatest diameter in prepubertal individuals and over 15 mm in greatest diameter in postpubertal individuals
- Two or more neurofibromas of any type or one plexiform neurofibroma
- Freckling in the axillary or inguinal regions
- Optic glioma
- Two or more Lisch nodules (iris hamartomas)
- A distinctive osseous lesion such as sphenoid dysplasia or thinning of the long bone cortex with or without pseudarthrosis
- A first-degree relative (parent, sibling, or offspring) with NF-1 by the above criteria

Prognosis

Compared to the healthy population, NF-1 patients exhibit a four times increased risk for malignant tumors, especially carcinomas and sarcomas. An analysis of death certificates in the USA revealed a decreased lifetime expectancy of 20 years compared to the general population with a mean age of death of 50 years for males and 54 years for females, respectively. The relative risk for connective and soft tissue carcinomas was increased by 34-fold. The incidence of pNENs including duodenal somatostatinomas is relatively low in NF-1patients [69]. However, in these cases, pNENs with NF-1 seem to include a great potential for malignancy.

Clinical Spectrum and Symptoms

Besides benign and malignant tumors of the peripheral and central nervous system, typical cutaneous manifestation such as café-au-lait spots and freckling of non-sun-exposed areas occur. Twenty-five percent develop an involvement of the gastrointestinal tract, the most common intestinal fibromas. Furthermore, pheochromocytoma (3–13%) and rarely pNENs including duodenal somatostatinomas (0–10%) have been reported to be associated to the disease [69, 70]. The gastrointestinal involvement which is observed in 25% of NF-1 patients includes hyperplasia of the plexus myentericus, neurofibromas, gastrointestinal stromal tumors (GIST), adenocarcinomas, pheochromocytomas, tumors of the papilla vateri, and pNENs. Klein et al. analyzed 37 VHL cases of periampullary neoplasms and found that the majority originates from the papilla (54%), followed by the duodenum (38%) and the pancreas (8%) [71].

Somatostatinoma is a distinct entity of periampullary neoplasms and mostly causes symptoms of duodenal obstruction such as jaundice, weight loss, abdominal pain, and gastrointestinal bleeding. A somatostatinoma syndrome related to a somatostatin excess with hyperglycemia, cholecystolithiasis, and imperfect digestion has yet not been reported in NF-1 patients.

Diagnostic Procedures

With regard to periampullary neuroendocrine tumor duodenoscopy, magnetic resonance cholangiopancreatography (MRCP) and EUS should be the first-line diagnostic tools followed by CT or MRI. Pancreatic neoplasms require EUS, MRI or CT scan, and SRS imaging (e.g., Ga-68 DOTATOC-PET/CT) as an adequate preoperative staging.

Treatment

Since pNENs in NF-1 patients are rare, recommendations for their treatment are only based on small case series and reach at most evidence level 4. Thus, the adequate

treatment of VHL-associated pNENs is still a matter of debate. If a tumor at the papilla vateri is smaller than 20 mm in size with no signs of metastatic spread after careful examination with endoscopy, EUS and MRI, a local excision, either endoscopically or surgically seem to be justified. In small pNENs (<20 mm), enucleation or parenchyma-sparing resections should be considered. pNENs larger than 20 mm or malignant spread requires distal pancreatectomy for pNENs located in the pancreatic tail and body or a PPD for pNENs located in the pancreatic head or the duodenum.

Screening and Surveillance

Screening in NF-1 patients has not been defined and general recommendations are lacking. Due to the low incidence of pheochromocytoma (0.1–5.7%) and pNENs (1%), regular screening is not generally recommended [68]. An expert panel and the Genetics Committee of the American Academy of Pediatrics have published diagnostic and health supervision guidelines for children with NF-1 in 2008 [72].

Conclusion

- MEN1

  Genetic screening and counseling are mandatory.
  Patients should be referred to specialized centers.
  Patients should be enrolled in regular screening programs.
  Gastrinoma is the most frequent functional pNEN.
  Malignant pNENs are the most common cause of death.

- Gastrinoma in MEN1

  Assess gastrin in every MEN1 patient.
  The duodenum is the target organ.
  Consider surgery in case of the biochemical diagnosis after exclusion of diffuse metastatic spread to provide a chance of cure.
  Duodenotomy with excision of all duodenal gastrinomas and lymphadenectomy are essential; PPPD is an alternative.

- Insulinoma in MEN1

  Preoperative localization is important for the operative strategy.
  EUS is superior to CT and MRI.
  Surgical treatment ranges from enucleation to partial pancreatectomy or distal pancreatectomy.
  Laparoscopic approaches are feasible.

- NF-pNENs in MEN1

  EUS is superior to CT and MRI.
  NF-pNENs are often multiple.
  Surgical treatment is indicated if the size exceeds 20 mm; in smaller tumors, surveillance is justified.
  Distal pancreatic resection and enucleation of pancreatic head tumors is the standard procedure, and enucleation for solitary tumors is an alternative.
  Laparoscopic approaches are feasible and safe.

Published Guidelines

- MEN1

  MEN1
  Clinical Practice Guidelines for MEN1, 2012 [1]
  ENETS Consensus Guidelines Update for the Management of Patients with Functional Pancreatic Neuroendocrine Tumors and Non-Functional Pancreatic Neuroendocrine Tumors, 2016 [48]
  National Comprehensive Cancer Center 2003 (www.nccn.org)

- VHL

  NCI (www.cancer.org)

- NF

  NIH consensus conference 1988 an update 1990 [55, 59]
  Health supervision for children with neurofibromatosis, 2008 [72]

Future Research Directions

- Prospective randomized multicenter trials are required to assess the use of regular screening on an EBM level.
- A general/worldwide accepted screening protocol for MEN1 patients.
- Establishing a genotype-phenotype correlation for MEN1-associated pNENs.
- Evaluation of pancreaticoduodenectomy as standard procedure for MEN1-associated ZES.

Cross-References

► Laparoscopic Surgery for Pancreatic Neoplasms
► Molecular Pathology of Carcinomas of the Ampullary/Periampullary Region

► MRI and MRCP for Diagnosis and Staging of Pancreatic Cancer
► Pancreatic Adenocarcinoma: CT and PET/CT
► Sporadic Pancreatic Endocrine Tumors

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Part II

Clinical Management of Pancreatic Cancer

Clinical Decision-Making in Pancreatic Cancer

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Contents

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Abstract

The management of pancreatic cancer relies on clinical staging for the majority of patients. High-quality cross-sectional imaging, and in some cases adjunctive staging modalities, partitions pancreatic cancer into one of four categories: localized and potentially resectable, borderline resectable, locally advanced/unresectable, or metastatic. Subsequent decisions regarding specific anticancer therapies and palliative interventions should be based on patient-centered, defined goals of care. Clinical decision-making should be evidence based, accounting for the patient's performance status and psychosocial circumstances, and developed with multidisciplinary input. Presently, surgical resection provides the only meaningful chance for long-term survival and, in general, is relevant only to those patients with potentially resectable or borderline resectable disease. However, there appears to be an expanding subset of patients with locally advanced disease who may eventually be considered surgical candidates. Nevertheless, surgical resection with curative intent should be linked to the delivery of additional therapy either as adjuvant therapy or neoadjuvant treatment. Enrollment in prospective clinical trials is always encouraged provided participation is not an undue burden on the patient or caregivers. Lastly, patients in need of expert clinical services should be encouraged to seek cancer care in pancreatic cancer centers of excellence as current evidence suggests improved outcomes in these settings.

Keywords

Resectable · Borderline resectable · Locally advanced · Metastatic · Adjuvant · Neoadjuvant · Gemcitabine · Nab-paclitaxel · FOLFIRINOX · Radiation

Introduction

Clinical decision-making as a disciplined exercise is not a new concept in medicine, or in oncology, yet its application to the management of patients with pancreatic cancer has previously been limited by the narrow range of available therapies. More recently, newer chemotherapeutic regimens and an expanding array of local therapies have provided a wider assortment of therapeutic options for all stages of this disease. Despite these advances, the prognosis for most patients with pancreatic cancer remains poor. Given the grim realities of pancreatic cancer, decision-making should be a shared endeavor, with a patient-centered focus. Importantly, as oncology care becomes increasingly multidisciplinary, clinicians from a variety of specialties must be aware of the assortment of interventions which may be utilized to minimize morbidity and toxicity, maximize palliation, and optimize patient survival. Furthermore, clinicians and patients alike face an intimidating challenge owing to the impressive dynamism of pancreatic cancer. This can result in the rapid onset of metastatic disease, local tumor progression associated with worsening pain or obstruction, venous thromboembolism, hemorrhage, or infection, especially involving the biliary tract. Clinicians must therefore be prepared for flexibility in clinical decision-making and to openly communicate how the goals of care may require sudden modification.

This chapter will be partitioned according to recognized clinical stages of pancreatic cancer and will attempt to provide a thoughtful, evidence-based approach to decision-making. Of note, any proposed interventions must be considered in the context of the patient's medical and psychosocial circumstances, and, whenever possible, multidisciplinary input should be sought prior to conclusive treatment planning. Moreover, enrollment in a clinical trial should always be encouraged, but understanding the potential burden of participation for the patient and caregivers is required.

Clinically Defined Stages of Pancreatic Cancer and Curative Potential

While some areas of controversy persist, there is emerging consensus that patients diagnosed with pancreatic cancer must undergo sufficient staging studies in order to classify them as having potentially resectable, borderline resectable (BR), locally advanced (LA), or metastatic disease. This allows oncologists to identify the minority of patients with localized disease with potential for curative therapy and to distinguish them from the larger group who present with non-curable disease.

Patients with localized pancreatic cancer, comprised of those with potentially resectable tumors and those with BR disease, have the greatest chance of cure or prolonged survival. Importantly, while surgery remains the only curative intervention, it can be morbid and lead to inadequate recovery which may impede or prevent the delivery of subsequent adjuvant therapy [1]. Of further note, if surgery is misapplied due to inadequate staging, it may nullify any meaningful chance for cure

[2]. If proper staging is utilized and its implications fully recognized, patients can be better informed of the options which may maximize their chances for prolonged survival or cure.

In general, patients with locally advanced pancreatic cancer (LAPC) do not have curative potential, but with appropriate management, durable local control and palliation can be achieved, and for many, the cancer's natural history can be altered [3]. For those presenting with metastatic disease, palliation should be paramount, and therapeutic options should not necessarily include the delivery of cytotoxic therapy. An open discussion of the goals of therapy for patients with established LA or metastatic disease should generally occur on the initial visit with the oncologist, and unrealistic expectations should not be endorsed or encouraged by any responsible physician.

Clinical Decision-Making in Patients Having Potentially Resectable Disease

Potentially resectable disease can only be defined if high-quality, dual-phase helical computed tomography (CT) or magnetic resonance (MR) imaging has been obtained. Resectable tumors do not involve critical venous or arterial structures, and furthermore, all imaging studies should have no evidence of distant metastatic disease. Resectable tumors should only be considered as such if there is a relatively high probability of an R0 resection (grossly and microscopically negative surgical margins).

For years, the standard of care for potentially resectable pancreatic cancer has been upfront resection, the most widely applied approach to date. Whenever possible, surgical resection should be followed by adjuvant therapy since modern clinical studies support a conclusive role for adjuvant therapy in patients who have undergone resection of the primary tumor with curative intent [4–6]. Some specific tenets about the role of upfront surgery for potentially resectable disease are enumerated below.

Seven Tenets for Potentially Resectable Pancreatic Cancer

1. Surgery at a high-volume center improves both short-term and long-term survival.
2. Surgery alone for resectable pancreatic cancer leads to poor survival.
3. Adjuvant therapy improves overall and 5-year survival after surgery with curative intent.
4. Upfront surgery is not always followed by the delivery of adjuvant therapy.
5. Preoperative performance status predicts the ability to receive postoperative therapy.
6. Preoperative CA 19-9 determinations are prognostic.
7. Positive surgical margins portend poor survival.

Surgery at a High-Volume Center Improves Both Short-Term and Long-Term Survival

There is an expanding literature to support the referral of patients with potentially resectable disease to centers that see a large number of pancreatic cancer patients [7, 8]. Analysis of SEER and Medicare databases in the USA also demonstrates better survival when patients receive therapy at academic medical centers, presumably representing high-volume university-based hospitals [9]. Furthermore, when pancreatic cancer surgical care is centralized, results suggest better overall outcomes [10]. Clinicians should therefore encourage patients to consider referral to a center of excellence whenever initial staging shows localized disease. While there may be socioeconomic factors which limit the feasibility of such a referral, the effort to make the referral or the time lag involved in the process should not be major impediments.

Surgery Alone for Resectable Pancreatic Cancer Leads to Poor Survival

Results from a number of studies and single-institution reports clearly demonstrate patients who undergo surgery as the only intervention for resectable pancreatic cancer have poor survival with early trials describing median overall survivals ranging from 10 to 13 months. More modern trials to include ESPAC-1, ESPAC-3 (v1), and CONKO 001 have all shown somewhat longer survival times for patients who were randomized to observation alone after surgical resection with curative intent with median survival times ranging from 17 to 20 months [5, 6]. These improved survival times likely reflect improvements in patient selection for surgery, surgical technique, and postoperative care in addition to better systemic therapy at the time of relapse. Nevertheless, the 5-year survival rate for patients who do not receive adjuvant therapy remains a dismal 10%. Thus, surgery alone is an inadequate strategy for patients with potentially resectable pancreatic cancer, and whenever a "surgery first" approach is being considered for a patient with resectable disease, it must be linked to the patient's potential to recover sufficiently to received postoperative therapy.

Adjuvant Therapy Improves Overall and 5-Year Survival After Surgery with Curative Intent

The data regarding adjuvant therapy for resected pancreatic cancer is discussed in greater detail elsewhere in the text. Beginning with ESPAC 1, originally reported in 2004, four large randomized trials of adjuvant therapy have established (a) there is no conclusive evidence that chemoradiation is a necessary component of adjuvant therapy, (b) systemic therapy improves survival over surgery alone, (c) gemcitabine monotherapy has equivalent efficacy and less toxicity compared with bolus

fluorouracil and folinic acid, and (d) the combination of gemcitabine with capecitabine leads to superior overall survival and 5-year survival compared with gemcitabine alone [4, 6, 11, 12].

At present, most authorities would consider upfront surgery and adjuvant therapy (now using gemcitabine and capecitabine) as the standard of care for patients with potentially resectable pancreatic cancer. However, as shown below, the data regarding upfront surgery and adjuvant therapy must be interpreted with caution.

Upfront Surgery Is Not Always Followed by the Delivery of Adjuvant Therapy

Five large adjuvant therapy trials have been completed and reported since 2004: ESPAC-1, RTOG 9704, ESPAC 3 (v2), CONKO 001, and most recently ESPAC 4 [4, 6, 11–13]. Together, these trials enrolled more than 3,000 patients. Unfortunately, the results from these trials have been limited to those patients who underwent an R0 or R1 resection and had adequate recovery from surgery to enroll in a clinical trial. None of these trials reported on the much larger number of patients who were taken to the operating room at the participating sites with plans to remove the primary tumor and deliver subsequent postoperative therapy. This is critical, since oncologists must recognize that of patients who present with potentially resectable pancreatic cancer and undergo surgery, slightly more than half go on to receive adjuvant therapy. Single-institution reports and analyses of large national databases show that only 50–60% of patients who undergo upfront surgery receive some form of adjuvant therapy [1, 9, 14]. The reasons for dropout from the time of surgery to administration of adjuvant therapy are likely related to three postoperative events. First is postoperative death. In an analysis of over 20,000 pancreatectomies registered in the National Cancer Data Base (USA), 30-day mortality was 3.7%; however, by 90 days, mortality increased to 7.4% [15]. Second are postoperative complications. In a separate analysis of over 2,000 pancreatectomies from the American College of Surgeons National Surgical Quality Improvement Program and the National Cancer Data Base, the delivery of adjuvant chemotherapy was only 58% [1]. The rate was 62% for patients with no significant complications and as low as 43% for those patients who developed at least one serious postoperative complication.

The third factor that likely precludes the delivery of adjuvant therapy is disease relapse in the immediate postoperative period. Unfortunately, the proportion of patients who undergo surgery with curative intent and subsequently develop overt metastatic disease during the usual recovery period from surgery (6–12 weeks) have not been rigorously analyzed or reported. However, in an analysis of seven previously published trials of neoadjuvant therapy for potentially resectable disease, 18% of enrolled patients developed radiographically detectable metastatic disease within 6–12 weeks from protocol enrollment [16]. It is therefore likely that of the patients who undergo pancreatectomy with curative intent, at least 15–20% will develop relapsing disease during their recovery period. Thus, when anticipated survival

results are discussed with patients prior to surgery, clinicians should be clear whether they are describing a "best-case scenario" or results that are applicable to the average patient since almost half do not receive postoperative therapy, a critical component of treatment beyond surgery.

Preoperative Performance Status Predicts the Ability to Receive Postoperative Therapy

While some have advocated surgical resection of primary pancreatic cancers as a palliative maneuver, survival of patients who undergo surgery as the only intervention is similar to the survival of patients with locally advanced, unresectable pancreatic cancer treated nonoperatively [3, 17]. Therefore, when surgery is being considered for a patient with potentially resectable disease, it should be realistically linked to that patient's potential to receive postoperative therapy. Preoperative clinical parameters which predict the likelihood of sufficient recovery to deliver postoperative therapy have not been well defined. Some data comes from a review of 85 patients undergoing upfront surgical resection for pancreatic cancer at the University of Texas, M.D., Anderson Cancer Center (UTMDACC) between 1994 and 2004 [18]. Three groups of patients were defined. Group 1 consisted of 13 patients who required emergent pancreaticoduodenectomy, group 2 had 63 patients having a preoperative Eastern Cooperative Oncology Group (ECOG) performance status (PS) 0 or 1, and group 3 was comprised of nine patients with ECOG PS of 2 or 3. Delayed recovery precluded the delivery of adjuvant therapy in 23% of patients in group 1, only 6% of those in group 2, and 44% of patients in group 3. Patients of advanced age (defined as >70 years) also appeared to have a lower chance of receiving postoperative therapy, but on multivariate analysis, only the urgency of surgery and the preoperative PS were identified as independent predictors of recovery which would allow for or preclude the delivery of postoperative therapy. Thus, when a patient presents with potentially resectable disease combined with compromised functional status, a decision to defer surgery is not unreasonable, and the option of initial nonoperative cancer-directed therapy is therefore an appropriate alternative.

Preoperative CA 19-9 Determinations Are Prognostic

The carbohydrate antigen 19-9 (CA 19-9) was initially characterized in a tumor cell line derived from a patient with colorectal cancer, and it can be elevated in a variety of malignancies. However, this serum tumor marker is frequently used to guide clinical care for patients with pancreatic cancer. In the setting of localized disease, preoperative CA 19-9 levels have been found to be prognostic. Patients who present with significantly elevated CA 19-9 levels despite radiographic evidence of potentially resectable disease have a worse prognosis compared with patients presenting with lower preoperative CA 19-9 levels. Unfortunately, published results will not

allow for an absolute cutoff to guide clinical decision-making, but in general preoperative CA 19-9 levels above 300 units/milliliter (U/mL) should lead to caution and possibly further staging evaluation prior to surgical intervention. For example, in a study reported by investigators in Liverpool, 159 patients who appeared to have resectable pancreatic cancer based on CT imaging underwent staging laparoscopy. Of the 63 patients with a preoperative CA 19-9 <150 U/mL, 60 (95%) had no evidence of metastatic disease at the time of laparoscopy, whereas only 78% of the 96 patients with a CA 19-9 above 150 U/mL were without metastatic disease [19]. Another study from Memorial Sloan Kettering Cancer Center lends further support to the use of preoperative CA 19-9 to increase the yield from laparoscopic staging using a cutoff of 130 U/mL [20].

After an analysis of data from the NCDB, the group from the Mayo Clinic has made a more controversial suggestion and advised neoadjuvant therapy for patients with potentially resectable pancreatic cancer having a serum CA 19-9 above the normal range [21]. In that study, the investigators divided patients who underwent surgical resection with curative intent into one of three groups: those with undetectable preoperative serum CA 19-9 levels, those with normal pre-op CA 19-9 levels, and those having elevated pre-op CA 19-9 levels. There was no difference in overall survival for those patients having undetectable or normal CA 19-9 levels pre-op. However, survival was inferior for those patients who presented with any elevation in CA 19-9 prior to surgical resection. The authors concluded that for patients who present with elevated preoperative CA 19-9 levels (after adequate biliary decompression), neoadjuvant therapy should be considered as initial therapy prior to surgical resection.

Surgeons should therefore consider using preoperative CA 19-9 determinations to identify patients who have radiographic evidence of potentially resectable pancreatic cancer, but whose preoperative CA19-9 level is sufficiently elevated to warrant staging laparoscopy or even to be referred for neoadjuvant therapy as initial treatment.

Positive Surgical Margins Portend Poor Survival

Over the past two decades, single-institution reports and results from large randomized trials have shown that microscopically positive surgical margins at the time of resection (R1) are associated with worse survival compared with the survival of patients undergoing an R0 (microscopically negative margins) resection [11, 12, 16]. This is irrespective of the delivery of postoperative therapy. These findings, coupled with improved imaging techniques, and reports of higher R0 resection rates for patients undergoing preoperative therapy have facilitated the recognition of BR disease. [22].

In summary, while surgical intervention for potentially resectable disease is always a desired goal, its benefits are limited if radiographically occult metastatic disease is present, an R0 resection is not achieved, or if subsequent adjuvant therapy cannot be delivered. Figure 1 depicts important parameters to consider in clinical decision-making which should initially limit the pool of patients advised to undergo

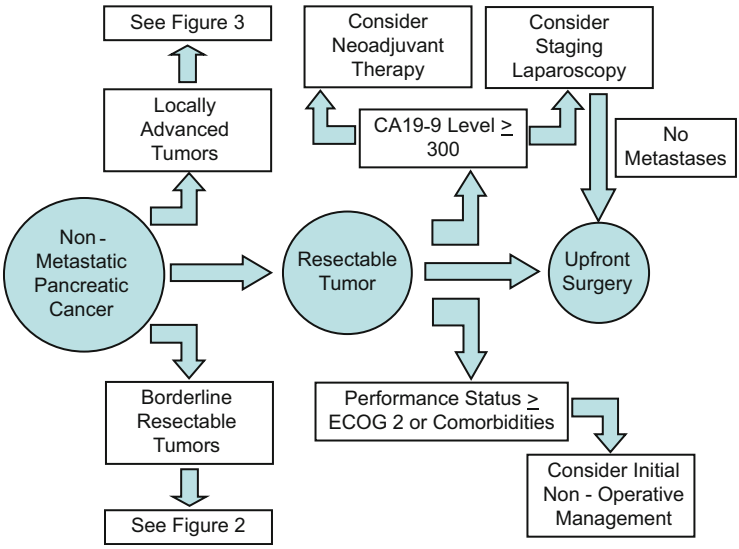

Fig. 1 Decision forks for patients with localized pancreatic cancer. The goal is to initially limit surgery to those patients at low risk for positive surgical margins, metastatic disease, or delayed post-operative recovery. Surgical decisions can be deferred for the remaining patients until after non-surgical therapies are delivered

immediate surgical resection. Patients with BR pancreatic cancer are by definition at high risk for positive surgical margins with upfront surgery, and under most circumstances, surgery should be deferred until some form of neoadjuvant therapy has been delivered as discussed below.

Clinical Decision-Making in the Setting of Borderline Resectable Disease

Over the last several years there has been growing recognition of a distinct subset of localized pancreatic cancers: those described as borderline resectable (BR) [22]. Three factors have led to this: the emergence of high-quality, dual-phase helical CT imaging and MR imaging, an onslaught of reports demonstrating the negative consequences of positive surgical margins on survival, and the ability of preoperative therapy to achieve higher rates of R0 resections [11, 12, 23, 24]. Detailed information on the definitions of BR disease can be found in the chapter entitled ▶ "Borderline Resectable Pancreatic Cancer". For the purposes of this chapter, a BR tumor is one which has a relatively high probability of a positive surgical margin if surgery is applied first. In clinical practice, BR pancreatic cancer needs to be recognized more frequently and fosters a discussion about preoperative therapy as an alternative to upfront surgical intervention. The four tenets listed below should inform clinical decision-making in patients with localized disease.

Four Tenets for Borderline Resectable Pancreatic Cancer

1. Positive surgical margins are frequent using upfront surgery in pancreatic cancer resections.
2. Borderline resectable tumors must be recognized.
3. Preoperative therapy for resectable pancreatic cancer leads to reduction in both the frequency of positive surgical margins and the risk of local failure.
4. Preoperative therapy should be considered as an alternative to upfront surgery in the setting of borderline resectable disease.

Positive Surgical Margins Are Frequent Using Upfront Surgery in Pancreatic Cancer Resections

Reports from the USA, Europe, and Asia all show that positive surgical margins occur among 37–60% of patients undergoing surgical resection with curative intent [12, 16]. Furthermore, although data suggest that postoperative therapy may provide some survival advantage compared with observation alone after a margin-positive resection, this rarely leads to cure [6]. These bleak statistics have focused attention on the need to achieve negative surgical margins at the time of resection to ensure any chance of long-term survival [2]. Since positive surgical margins with upfront surgery virtually eliminate any meaningful chance of cure, surgery as the initial intervention for a localized cancer should be limited to tumors which can be removed with a high probability of negative surgical margins. In centers which rely on strict radiographic criteria to define resectable disease, the rate of positive surgical margins with upfront surgery is around 20% [25].

Borderline Resectable Tumors Must Be Recognized

If BR disease is not recognized by the surgeon and radiologist, upfront surgical resection is likely to result in an R1 resection, and any meaningful chance for long-term survival is lost. If, however, high-quality preoperative imaging is acquired and properly interpreted, a subset of patients will be recognized as having BR tumors and better informed as to the choices between immediate resection and deferral of surgical intervention until preoperative therapy has been delivered. Therefore, radiologists and surgeons must confer on the results of high-quality cross-sectional imaging with attention to evidence for tumor-vessel abutment.

Preoperative Therapy for Resectable Pancreatic Cancer Leads to Reduction in Both the Frequency of Positive Surgical Margins and the Risk of Local Failure

In the setting of potentially resectable disease, preoperative therapy remains investigational. However, survival results using preoperative therapy are at least equivalent, if

not superior to upfront surgery and adjuvant therapy. Preoperative therapy has three significant potential advantages compared with upfront surgery and postoperative therapy. First, it provides a selection mechanism to identify those patients with resistant and rapidly progressive disease who will not benefit surgery. In trials conducted to date, approximately 18% of patients develop radiographically evident metastatic disease in the face of preoperative therapy [16]. Second, single-institution experience with preoperative therapy suggests rates of positive surgical margins as low as 6–11% [26, 27]. Third, local failure rates are lower using preoperative therapy than those reported with a surgery-first approach [28]. Importantly, isolated local tumor progression which precludes surgery after preoperative therapy is rare. [26, 27].

Preoperative Therapy Should Be Considered as an Alternative to Upfront Surgery in the Setting of Borderline Resectable Disease

Although a localized rectal cancer has less propensity to disseminate compared with localized pancreatic cancer, the radial or circumferential margin of a rectal cancer has similarities to the retroperitoneal or superior mesenteric artery (SMA) margin in pancreatic cancer (the margin most likely to be positive after a pancreaticoduodenectomy) [25]. When either the radial margin is positive in rectal cancer or the SMA margin is positive in pancreatic cancer, the patient is placed at higher risk for relapse and death compared with an R0 resection [2, 29]. A large body of literature in rectal cancer and a more modest data set in resectable pancreatic cancer both suggest that preoperative therapy, specifically involving radiation, reduces the risk of a positive surgical margin and local failure [24, 28].

These principles are now being applied to patients with BR pancreatic cancer. Retrospective data have been available for some years. In a report from UTMDACC, three groups of borderline patients were defined: those having radiographic evidence of a tumor that was borderline for resection (group A), those whose preoperative imaging had equivocal evidence of metastatic disease (group B), and those patients whose physiologic status or medical comorbidities put them at increased risk for postoperative complications or hampered recovery [30]. Of the 84 patients in group A, 32 (38%) ultimately underwent surgery with curative intent after preoperative therapy, with all but one undergoing an R0 resection. The survival for the subset ultimately undergoing resection was impressive with a median overall survival of 40 months. Other groups have also reported on the ability of preoperative or neoadjuvant therapy to convert marginally resectable or locally advanced pancreatic cancer to resectable disease [31]. Recently, the utilization of modern chemotherapy regimens for BR disease has begun to appear in the literature. Two groups have reported on the use of folinic acid, fluorouracil, irinotecan, and oxaliplatin (FOLFIRINOX) or modified FOLFIRINOX as induction chemotherapy followed by chemoradiation to allow for resection rates between 61% and 67% in their patients with BR disease [32, 33]. Among the resected patients, R0 margins were achieved for 82–100%. Most recently, a multi-institutional trial of FOLFIRINOX and capecitabine-based chemoradiation delivered to 22 patients has been published

[34]. The overall resection rate was 68% with all but one patient undergoing an R0 resection. Of further note, 33% of resected specimens had <5% viable tumor present. (Survival data from this trial are not available yet.)

There is less information on the use of gemcitabine and nab-paclitaxel as neoadjuvant therapy for borderline resectable or locally advanced pancreatic cancer. However, this regimen has been reported as an acceptable regimen for preoperative therapy in resectable pancreatic cancer [35]. For patients with BR or LAPC, an Italian phase I trial has reported on a combination of gemcitabine, nab-paclitaxel, capecitabine, and cisplatin as having potential to downstage tumors to surgical resection [36]. Among the 25 patients enrolled, six underwent surgical resection with an R0 resection rate of 50%. Whether radiation is a necessary component of neoadjuvant therapy for BR pancreatic cancer will require well-conducted randomized clinical trials. Nevertheless, taken together, the results above support the principle that preoperative therapy can be sufficiently destructive to tumor, to ultimately allow for R0 resections in patients previously defined as having BR disease.

Clinicians may be concerned that if no attempt is made to resect a BR tumor with upfront surgery, the window of opportunity to do so may be lost. However, if surgery proceeds and the patient is left with microscopic or macroscopic tumor, the postoperative prognosis is poor. Figure 2 depicts a decision tree that emphasizes neoadjuvant chemotherapy, chemoradiation, or both with subsequent restaging studies prior to consideration of surgical intervention.

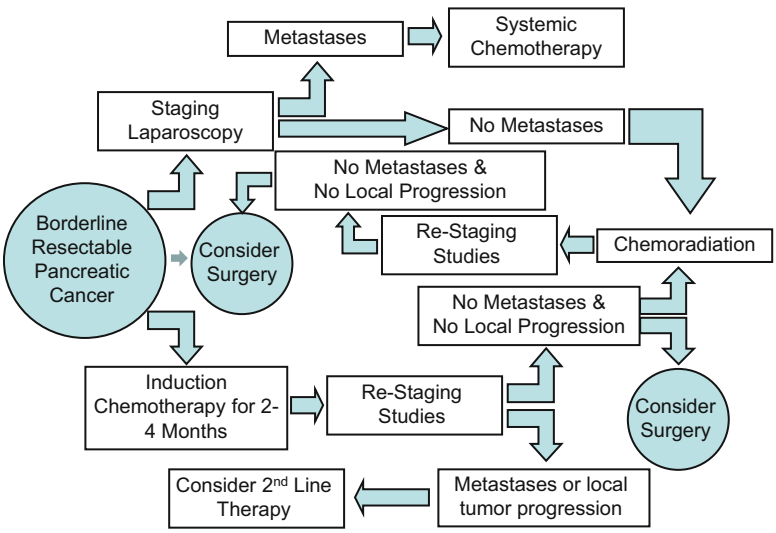

Fig. 2 Decision-tree for patients with borderline resectable disease. While no standard approach exists, neoadjuvant chemotherapy, chemoradiation, or both is generally recommended over upfront surgery

Importantly, the response criteria used to support attempt at surgical resection after deliver of neoadjuvant therapy remains poorly defined and currently relies on clinical parameters (such as reduction in pain or drop in serum CA19-9 levels). Reports are beginning to emerge in the literature that the use of radiographic criteria to determine resectability after a period of neoadjuvant therapy is a challenge (with the exception of interval development of metastases) [37].

Although the delivery of preoperative therapy for potentially resectable pancreatic cancer is encouraged in the context of a clinical trial, the data regarding preoperative therapy is sufficiently compelling to consider it for patients defined as having BR disease. The National Comprehensive Cancer Center Network (NCCN) pancreatic cancer subcommittee recommends preoperative therapy as preferred over upfront surgery in the setting of BR pancreatic cancer [38]. However, preoperative therapy for BR disease is not endorsed by the International Study Group of Pancreatic Surgery unless it is delivered as part of a clinical trial [39].

Decision Analysis in Patients with LAPC

The majority of patients staged with locally advanced pancreatic cancer have incurable disease, and all clinical decisions should keep palliation as a top priority. Furthermore, locally advanced tumors often put patients at risk for local invasion to include gastric outlet obstruction, biliary obstruction, and portal vein thrombosis. Thus, clinicians should be mindful of local control even when interventions intended to address it do not necessarily prolong survival. Currently, management strategies for locally advanced pancreatic cancer fall into two categories: cancer-directed therapies and palliative interventions to include surgical bypass, endoscopic and percutaneous procedures, and other supportive measures. Fortunately, these interventions are not mutually exclusive, and when appropriate, patients may embark on cancer-directed therapies as other palliative interventions are considered as components of care. However, clinicians should not recommend cancer-directed therapy until the patient is sufficiently stable to do so. Initiating potentially toxic therapy in patients with dynamic clinical status or poorly controlled symptoms is often counterproductive and should be discouraged, even when the patient or caregivers are anxious to proceed.

At the time of initial consultation, a thorough appraisal of the patient's social support, prior and current functional status, nutrition, and symptoms should be completed. In addition, a careful physical examination may uncover findings not evident from laboratory or radiographic studies to include the discovery of Virchow's nodes or the presence of superficial or deep venous thromboses. Immediate problems should be addressed during that visit, and although treatment options may be discussed, clinical decisions about cancer-directed therapy may need to be deferred until better symptom control is achieved.

The management of locally advanced disease has been in evolution over the last 10–15 years. In an earlier era, fluorouracil-based chemoradiation was often the initial treatment. Thereafter, numerous clinical trials investigated other chemoradiation

regimens, with the majority leading to median survival durations of 10–12 months. However, with the approval of gemcitabine for the treatment of patients with locally advanced or metastatic pancreatic cancer, the delivery of radiation as a necessary component of treatment for locally advanced disease became an open question. Results from two randomized trials (one in Europe and one in the USA) offered conflicting results. European investigators questioned whether chemoradiation followed by gemcitabine was superior to gemcitabine alone for the treatment of patients with locally advanced disease [40]. This trial randomized 119 patients to receive an intensive course of chemoradiation with 5-FU and cisplatin followed by post-chemoradiation gemcitabine or to receive gemcitabine monotherapy alone. The results showed that the intensive chemoradiation program with subsequent gemcitabine was more toxic than the delivery of gemcitabine alone. In addition, median survival for patients randomized to chemoradiation and subsequent gemcitabine was only 8.6 months; this was inferior to the survival of patients treated with gemcitabine alone (13 months, $p = 0.03$). Given the toxicity associated with the intensive chemoradiation, it may be that the worse survival of the patients randomized to receive chemoradiation was a reflection of toxicity and not superior efficacy using single-agent gemcitabine.

In a similar fashion, ECOG conducted a trial in which 74 patients were randomized to receive gemcitabine alone or gemcitabine plus radiation followed by gemcitabine [41]. Patients who were randomized to gemcitabine plus radiation had a median survival of 11.4 months which was statistically superior to the median survival of those patients randomized to gemcitabine alone (9.6 months, $P < 0.03$). However, the survival curves revealed that the addition of radiation to gemcitabine only provided a survival advantage to those patients surviving more than 6 months; there was no difference in survival between the two arms for patients with shortened survival. These results implied that local control was only relevant to the subset of patients with more favorable tumor biology, and thus induction chemotherapy might provide a selection mechanism to identify patients with rapid onset of metastatic disease and distinguish them from a larger subset of patients more likely to benefit from follow-on chemoradiation. Retrospective studies and prospective clinical trials appeared to support this approach, and median survivals in these publications ranged from 12 to 19 months. Of note, during the period of induction chemotherapy, roughly 30% of patients manifest aggressive tumor biology which precluded the subsequent delivery of radiotherapy. This subset of patients was observed to have poor survival.

Very recently, however, the paradigm of induction chemotherapy followed by chemoradiation as an optimal strategy has been refuted [17]. In LAP07, a large international, multicenter trial coordinated by the Groupe Coopérateur Multidiciplinaire en Oncologie (GERCOR), patients who remained progression free after 4 months of gemcitabine monotherapy or gemcitabine plus erlotinib were subsequently randomized to continue gemcitabine +/− erlotinib for two additional months or to switch to treatment with capecitabine-based chemoradiation. Radiation was delivered to a total dose of 54 Gy. There was no significant difference in median survival between those who continued gemcitabine +/− erlotinib (16.5 months) and

those who received follow-on chemoradiation (15.2 months). Of note, fairly consistent with earlier trials of induction chemotherapy, 40% of the initial cohort of patients dropped out during the first 4 months of induction chemotherapy prior to randomization to continued chemotherapy versus a switch to chemoradiation, predominantly based on progressive disease. These results, combined with findings from other studies highlighted below, lead to the following tenets in regard to clinical decision-making for patients with locally advanced disease.

Five Tenets for Locally Advanced Pancreatic Cancer

1. Laparoscopy has a limited role in the current staging of patients with LAPC.
2. Patients staged with locally advanced pancreatic cancer and having adequate PS with manageable symptoms should undergo initial treatment with systemic therapy.
3. Local therapeutic strategies may have a role in management after a period of systemic therapy for subsets of patients with LAPC.
4. Surgical resection with curative intent is possible for some patients with LAPC.
5. Options for less invasive or nonoperative palliation of biliary obstruction and gastric outlet obstruction are expanding.

Laparoscopy Has a Limited Role in the Current Staging of Patients with LAPC

High-quality cross-sectional body imaging has allowed for more accurate staging of pancreatic cancer, and the yield of staging laparoscopy appears to be decreasing over time. Nevertheless, some experts have advocated laparoscopy as a routine staging procedure. Several studies have shown that laparoscopy can upstage a subset of patients with locally advanced disease by visualizing small surface liver metastases or peritoneal implants or cytologic examination of peritoneal washings. However, with systemic therapy now the standard of care for patients with locally advanced cancer, the documentation of radiographically occult metastases becomes less relevant. Furthermore, if a patient subsequently develops radiographic or other clinical evidence of metastatic disease after induction chemotherapy, the need for a staging laparoscopic examination diminishes further. Currently, staging laparoscopy is most appropriate for patients in whom local therapies (radiotherapy or ablative techniques) are being considered after an initial period of systemic chemotherapy. In these circumstances, laparoscopy may indeed impact clinical decision-making.

Importantly, there may be clinical situations in which local interventions may be justified even when small volume metastatic disease is evident or suspected. These would include bleeding from ulcerated gastrointestinal mucosa infiltrated with tumor, intractable pain not responsive to medical management or neurolytic plexus block, and possibly when there appears to be an increased risk of gastric outlet

obstruction secondary to tumor encroachment on the duodenum, or recurrent biliary obstruction related to tumor ingrowth.

Patients Staged with Locally Advanced Pancreatic Cancer and Having Adequate PS with Manageable Symptoms Should Undergo Initial Treatment with Systemic Therapy

Based on the results from LAP07, there is no survival advantage to consolidating conventional chemoradiation after an initial period of induction chemotherapy. While some might argue that the available data supports the delivery of gemcitabine monotherapy as the standard regimen, various investigators have begun to report on the administration of either FOLFIRINOX or gemcitabine/nab-paclitaxel as frontline therapy for patients with locally advanced disease [42, 43]. Currently, there is no definitive evidence to prefer one systemic regimen over another in the setting of locally advanced disease [44]. However, there may be distinct subsets of patients with locally advanced disease who may be candidates for more aggressive combination regimens rather than treatment with gemcitabine alone. These may include very fit patients with no comorbidities or contraindications to treatment with a platinum-containing regimen such as FOLFIRINOX. Conversely, both FOLIRINOX and gemcitabine/nab-paclitaxel may be less attractive initial options for patients with long-standing diabetes or having peripheral neuropathy from other causes. Moreover, given that the majority of patients with locally advanced disease will develop metastatic disease, delivering FOLFIRINOX as initial therapy will limit therapeutic options at the time of progression.

Lastly, although relatively few, there are distinct patients with locally advanced disease in whom an aggressive multimodal approach may provide an opportunity for prolonged local control and even ultimate surgical resection with curative intent. Such patients represent a minority of those who present with locally advanced disease, and their management will be discussed below.

Local Therapeutic Strategies May Have Role in Management After a Period of Systemic Therapy for Subsets of Patients with LAPC

LAP07 demonstrated no survival advantage using chemoradiation after 4 months of systemic gemcitabine compared with two additional months of gemcitabine. However, there were some clinical benefits for those patients who were randomized to receive chemoradiation. First, local tumor progression was decreased with chemoradiation compared to continued systemic therapy (32% vs 46%). Second, in both arms of the trial, all therapy was discontinued after a total of six cycles of gemcitabine or 4 months of gemcitabine and subsequent chemoradiation. For those randomized to chemoradiation, there was a longer chemotherapy-free interval prior to resumption of additional chemotherapy (6.1 months vs 3.7 months, $p = 0.02$). At present, the American Society for Clinical Oncology recommends radiation only for

those patients who have local tumor progression (without evidence of metastases) after a period of induction chemotherapy [44]. In addition, radiation may be an alternative to continued chemotherapy for those patients with LAPC, who develop intolerable side effects to chemotherapy. However, for patients with very favorable tumor biology and a durable response to induction chemotherapy, consolidation with locally ablative therapies may provide a longer chemotherapy-free interval and improve quality of life.

Importantly, the options for local therapies are expanding and now include radiation given as stereotactic body radiotherapy (SBRT), microwave ablation (MA) or radiofrequency ablation (RFA), and irreversible electroporation (IRE) as an alternative or adjunct to radiotherapy for patients with LAPC. SBRT, MA, RFA, and IRE are attractive technologies in that their delivery is of short duration and allow the patient to resume systemic therapy relatively quickly. In addition, these modalities may provide longer more durable chemotherapy-free intervals to enhance quality of life.

Phase II trials of SBRT in LAPC are now appearing in the literature with doses ranging from 33 Gy up to 45 Gy with most patients previously treated with induction chemotherapy [45, 46]. Survival durations with SBRT appear comparable if not superior to those reported in LAP07. These results suggest that SBRT given in five to six fractions is better tolerated and more convenient than standard chemoradiation, with similar efficacy.

In the future, delivery of SBRT after a 4–6 month period of systemic therapy with no interval development of metastatic disease may offer the advantage of a relatively brief intervention for improved local control that can soon be followed by a return to systemic therapy, or alternatively, a period of observation, and for a small, select subset, surgical resection [47].

IRE is a locally ablative strategy that does not lead to thermal injury to surrounding tissues, specifically vascular and ductal structures. The largest experience with IRE in localized pancreatic cancer comes from a multi-institutional trial conducted by the University of Louisville. Investigators there reported on their experience with IRE in 54 patients with pancreatic cancer (90% of whom were previously treated with systemic therapy) and suggested this intervention had the potential to prolong survival over traditional strategies utilizing systemic therapy and chemoradiation [48]. Other centers are beginning to report on their experience with IRE to include percutaneous, image-guided localization of the electrodes [49].

Other locally ablative techniques (MA and RFA) are being reported in the medical literature in more limited fashion. Based on current literature, however, there appears to be growing enthusiasm for IRE, particularly for patients with BR pancreatic cancer or LAPC [50]. However, as previously emphasized, given the propensity for metastases, systemic therapies should precede any local therapeutic intervention (chemoradiation, RFA, SBRT, IRE, or surgery). Moreover, experience with SBRT and locally ablative techniques is currently limited to a few centers, and well-conducted clinical trials are needed to better define the role of these technologies in the future management of patients with locally advanced pancreatic cancer. Nevertheless, these newer modalities are certain to be refined, and their incorporation into the management of some patients with LAPC is anticipated.

Surgical Resection with Curative Intent Is Possible for Some Patients with LAPC

Current criteria to define LAPC generally include tumors with >180° involvement of the SMA or celiac trunk. In an earlier time period, such tumors were rarely downstaged to the point of resectability after treatment with neoadjuvant therapy. However, the growing number of reports of successful resection of BR tumors after neoadjuvant therapy (with encouraging survival durations), coupled with the availability of more active systemic cytotoxic regimens, has led to recent reports of successful resection of pancreatic adenocarcinomas previously considered locally advanced and unresectable. In general, reports of neoadjuvant therapy to downstage LAPC have relied on initial systemic therapy followed by a local therapy (conventional radiotherapy, SBRT, or IRE).

For example, investigators at Moffitt Cancer Center reported on 159 patients with BR (110) or LAPC (49) [47]. Among the patients with LAPC, 21 received FOLFIRINOX as induction therapy, and 28 received various other chemotherapy regimens. Among the patients treated with FOLFIRINOX, five (24%) ultimately underwent an R0 resection after further therapy with SBRT. None of the patients treated with other systemic regimens underwent an R0 resection. Of further note, among the patients with BR or LAPC who underwent R0 resection, median survival was 34.2 months.

In another report from the group at the Johns Hopkins Hospital, patients who underwent a distal pancreatectomy were matched 3:1 to patients who had celiac axis encasement requiring celiac axis resection as a component of the distal pancreatectomy (modified Appleby's procedure) [51]. Of the patients who underwent celiac axis resection/distal pancreatectomy, 88% initially underwent neoadjuvant therapy usually with FOLFIRINOX and SBRT. There was no difference in survival between those requiring a modified Appleby's and those who underwent distal pancreatectomy alone implying that aggressive neoadjuvant multimodal therapy may expand the proportion of patients with LAPC who are eligible for ultimate resection with curative intent.

Further evidence that modern systemic chemotherapy may facilitate tumor downstaging in LAPC comes from a systematic analysis of patients treated with FOLFIRINOX [52]. This study involved 365 patients with LAPC treated with FOLFIRINOX, of whom 57% also received radiation. Of the 365 patients, 103 (28%) underwent subsequent resection with an R0 rate of 77%. Of note, among those patients treated with FOLFIRINOX alone (without radiation), only 12% went on to surgical resection (70% R0) suggesting radiotherapy may be of additional benefit in downstaging LAPC.

In addition to more aggressive combination chemotherapy with or without subsequent radiation, intraoperative IRE as an adjunct to surgical resection in BR or LAPC is also being explored. Investigators at the University of Louisville have described the potential of IRE to provide for "margin accentuation," thereby increasing the chances of an R0 resection for patient initially staged as having BR or LAPC [53].

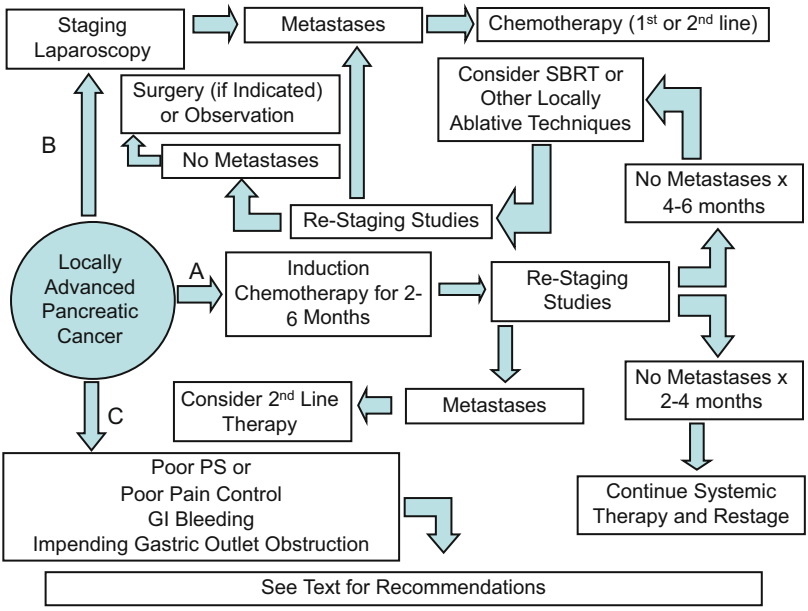

Fig. 3 Decision pathway for patients with LAPC. Pathway (**a**) utilizes induction chemotherapy without need for staging laparoscopy. Pathway (**b**) utilizes laparoscopy to upstage some patients. Pathway (**c**) is for the small group of patients with poor PS or immediate local control problems irrespective of the presence of low-volume metastatic disease

In summary, as systemic therapy for pancreatic cancer improves and various ablative techniques are further developed, an expanding subset of patients with LAPC may be considered for curative surgical resection. Thus, for a patient with LAPC having good PS and a stable or responding primary tumor (with no interval metastatic disease after 4–6 months of systemic therapy), referral to a center of excellence should be encouraged. See Fig. 3 below for a more detailed decision tree.

Options for Less Invasive or Nonoperative Palliation of Biliary Obstruction and Gastric Outlet Obstruction Are Expanding

Although palliative interventions are appropriate for patients with all stages of pancreatic cancer, clinical decision-making, particularly as it pertains to palliative surgery, remains a challenge. In years past, many patients underwent exploratory laparotomy for potentially resectable disease with intraoperative discovery of radiographically occult metastatic disease or unresectable tumor. While most surgeons would agree with operative biliary bypass for unresectable patients undergoing exploratory laparotomy, for tumors in the head of the pancreas, there is no consensus about prophylactic gastrojejunostomy. Therefore, prophylactic gastrojejunostomy should be left to the surgeon's best judgement considering the extent of local disease, the metastatic

tumor burden encountered, comorbidities, and the patient's life expectancy. Of note, this clinical decision is probably less relevant today, and the role of palliative surgery for patients with pancreatic cancer appears to be waning. There are three reasons for this. First, better preoperative imaging is now distinguishing potentially resectable tumors from borderline resectable and locally advanced tumors; the latter two categories have more limited indications for initial surgery. Second, high-quality imaging also appears to be improving the detection of low-volume metastatic disease. Third, these imaging advances occurring as nonoperative interventions to address biliary and gastric outlet obstruction are also expanding.

For patients with LAPC, where prognosis is intermediate to resectable and metastatic disease, the array of options to manage biliary obstruction include surgical bypass, endoscopic biliary stenting, and percutaneous biliary decompression with or without transhepatic deployment of a stent [54]. Previously, nonoperative approaches were often not durable or required periodic stent or catheter exchanges related to occlusion and ongoing potential for cholangitis. With the availability of self-expanding metal stents (covered or uncovered) which can be inserted by either a transhepatic or an endoscopic approach, durable biliary drainage can be accomplished for a growing proportion of patients. Among patients with LAPC, the management of bile duct obstruction may be more complicated, especially for those with longer life expectancy. In general, nonsurgical approaches are durable for most patients, although some may require metal stent revision, usually related to occlusion from debris or tumor ingrowth.

The management of gastric outlet obstruction also requires careful deliberation. This complication may occur in isolation, but is often accompanied by disease progression beyond the primary tumor. Here too, options for management are expanding and include open gastrojejunostomy, laparoscopic gastrojejunostomy, duodenal stenting, and, for some patients, decompressive gastrostomy tube. For surgical candidates, laparoscopic gastrojejunostomy appears to be at least equivalent to open gastrojejunostomy in terms of length of hospital stay and resumption of oral feeding. Surgical intervention should be limited to patients having local tumor progression without evidence of metastatic disease or having very limited metastatic tumor burden. The presence of ascites or peritoneal disease would be relative contraindications to surgical intervention. For patients with documented metastatic disease, particularly those having progressive metastases, or otherwise considered as poor surgical candidates, insertion of a duodenal stent appears to be safer, more effective, and less costly. There is a small group of patients with functional outlet obstruction or multifocal bowel obstruction due to intra-abdominal metastases. For these patients endoscopic or percutaneous insertion of a decompressing gastrostomy tube may be more appropriate.

Whenever possible, patients with locally advanced pancreatic cancer should be referred to a center of excellence in pancreatic cancer in order to develop an initial strategy for symptom management and anticancer therapy. For patients with jaundice, decompression of the biliary tree should occur prior to the delivery of cytotoxic therapy. Whether oncologic therapy is subsequently delivered in a community clinic or a tertiary center, frequent follow-up to monitor toxicities of treatment, signs and

symptoms of cholangitis, venous thromboembolism, pain, hemorrhage, or onset of gastric outlet obstruction is required. Moreover, for patients who develop complex local control problems, multidisciplinary input from surgeons, gastroenterologists, interventional radiologists, and oncologists should be encouraged. This is also important for the small number of patients who may benefit from other technically advanced procedures such as portal venous stenting or neurolytic pain blocks performed using a percutaneous image-guided approach or under endosonographic guidance [55–57].

Clinical Decisions in Metastatic Pancreatic Cancer

Since the last edition of this text, the chemotherapeutic options for patients with metastatic disease have expanded, and overall, expected survival for patients with metastatic disease and adequate PS have improved modestly. Three new regimens have been developed and approved for patients with metastatic disease: FOLFIRINOX, gemcitabine/nab-paclitaxel, and, as a second-line treatment, nanoliposomal irinotecan (nal-iri) administered with folinic acid and fluorouracil (FF) [58–60]. These drug combinations were approved based on large randomized clinical trials with varying patient eligibility criteria, and these variations should be appreciated in clinical decision-making. FOLFIRINOX was studied in previously untreated patients with enrollment limited to patients with ECOG PS 0 or 1 [58]. The trial demonstrated a survival advantage for treatment with FOLFIRINOX over gemcitabine (11.6 months vs 6.7 months, respectively, $p = 0.002$). The Metastatic Pancreatic Cancer Trial (MPACT) which led to the approval of nab-paclitaxel as frontline therapy in combination with gemcitabine had more relaxed eligibility criteria and allowed trial entry for patients with Karnofsky Performance Status (KPS) $\geq$70% (roughly equivalent to ECOG $\leq$2) [59]. MPACT demonstrated a survival advantage for patients randomized to gemcitabine/nab-paclitaxel compared with those who received gemcitabine alone (median overall survival 8.5 months vs 6.7 months, $p = 0.001$). Lastly, nanoliposomal irinotecan (nal-iri) was approved for use in second-line therapy for patients who had failed initial therapy with gemcitabine alone. In a trial which enrolled 417 patients, the combination of FF with nal-iri led to a median survival of 6.1 months compared with 4.2 months for patients who received FF (hazard ratio for death 0.67, $p = 0.012$) [60].

When considering treatment it must be recognized that patients with metastatic pancreatic cancer often present with significant symptom burden and marginal functional status for cytotoxic therapy. Such patients generally have poor survival, and importantly, some analyses suggest that combination therapy may be detrimental to survival compared with less aggressive therapy. Therefore, questions to be posed in decision-making include whether or not systemic therapy should be advised, how aggressive it should be, and, at some point, if second-line therapy should be offered. With this in mind, some general tenets of anticancer therapy are discussed below.

Five Tenets for Patients with Metastatic Pancreatic Cancer

1. Chemotherapy prolongs survival over best supportive cancer in patients with advanced pancreatic cancer.
2. Patients with good performance status benefit from combination chemotherapy over treatment with gemcitabine alone.
3. Patients with poor performance status may do worse with combination chemotherapy compared with monotherapy and may not benefit from the delivery of any cytotoxic therapy.
4. Second-line therapy may be appropriate for some patients who progress after frontline therapy.
5. Surgery or other noninvasive ablative strategies may be relevant for a small subset of stage IV patients with limited metastatic and/or persistent local disease after initial systemic therapy.

Chemotherapy Prolongs Survival Over Best Supportive Cancer in Patients with Advanced Pancreatic Cancer

In years past, chemotherapy trials using older regimens have shown improvements in survival compared with best supportive care. A meta-analysis of several trials demonstrated a clear survival benefit for patients treated with systemic therapy compared with those receiving best supportive care [61]. This analysis, which included 51 trials and 9,970 enrolled patients, showed improved survival with chemotherapy (hazard ratio = 0.64; 95% CI, 0.42–0.98). At present, however, it remains somewhat uncertain what level of functional status is necessary for a patient to actually benefit from the delivery of cytotoxic therapy. As will be discussed later, some results suggest that patients with poor PS may not receive any meaningful benefit from cytotoxic chemotherapy, and others imply that more aggressive combination therapy may be detrimental to survival compared with treatment using monotherapy.

Patients with Good Performance Status Benefit from Combination Chemotherapy Over Treatment with Gemcitabine Alone

As discussed above, for patients with metastatic disease having ECOG PS 0–1, FOLFIRINOX is clearly superior to gemcitabine alone in terms of objective response rate and overall survival. The same is true for gemcitabine plus nab-paclitaxel for patients with KPS $\geq$70%. Of interest, the MPACT investigators did an analysis of overall survival based on KPS score on trial entry. For patients with KPS >80%, the median survival for patients treated with gemcitabine/nab-paclitaxel was 9.7 months, whereas for patients with KPS 70–80%, the median survival was only 7.6 months [62]. Clinicians should therefore recognize that the benefits of combination therapy are more robust when reserved for patients with well-preserved performance status. This is supported by an earlier analysis of five

randomized trials comparing gemcitabine doublets to gemcitabine monotherapy and suggested that a survival advantage with combination therapy was only conferred on those patients with KPS >80% [63].

Patients with Poor Performance Status May Do Worse with Combination Chemotherapy Compared with Monotherapy and May Not Benefit from the Delivery of Any Cytotoxic Therapy

The link between performance status and survival in patient with pancreatic cancer has been known for decades. Although FOLFIRINOX and gemcitabine/nab-paclitaxel are more active combinations, they are also more toxic compared with gemcitabine monotherapy. Thus, careful evaluation of a patient's PS is critical for informed decision-making. For example, although a survival advantage was observed using gemcitabine/nab-paclitaxel among patients with KPS = 70%, the median survival of this subgroup was quite poor using either the doublet or gemcitabine monotherapy (3.9 months vs 2.8 months, respectively) [62]. Further evidence for caution in advising patients with marginal PS comes from the subset analysis of the randomized trials of other gemcitabine doublets versus gemcitabine alone. This analysis suggested that for those patients with poor performance status (KPS <80%), combination therapy led to worse survival compared with the delivery of gemcitabine alone [63]. This result is not surprising, and while not definitive, more aggressive, toxic therapy may be detrimental for poor PS patients. Furthermore, whether any cytotoxic treatment is beneficial for patients with marginal PS is questionable. Sobering data comes from a randomized trial conducted by the Cancer and Leukemia Group B which compared gemcitabine and bevacizumab to gemcitabine plus placebo in patients with advanced pancreatic cancer. There was no difference in survival between the group treated with gemcitabine and bevacizumab and the group assigned to gemcitabine and placebo [64]. Importantly though, there was a clear difference in survival based on patient performance status at study entry. Those patients reported to have an ECOG PS of 0 had a median survival of 8 months, while those with ECOG PS 1 had a median survival of 4.8 months, and patients with an ECOG PS 2 had a median survival of only 2.8 months. Although some of the patients reported to have an ECOG PS of 2 probably had an ECOG PS closer to 3, a median survival less than 3 months suggests no meaningful benefit from the delivery of chemotherapy. Clinicians should therefore be cautious when facing a patient with poor PS, and any inclination to offer cytotoxic therapy should be tempered by this data (Fig. 4).

Second-Line Therapy May Be Appropriate for Some Patients Who Progress After Frontline Therapy

Although it is common for disease progression to be associated with worsening PS, there is a subset of patients who will maintain sufficient PS to consider second-line

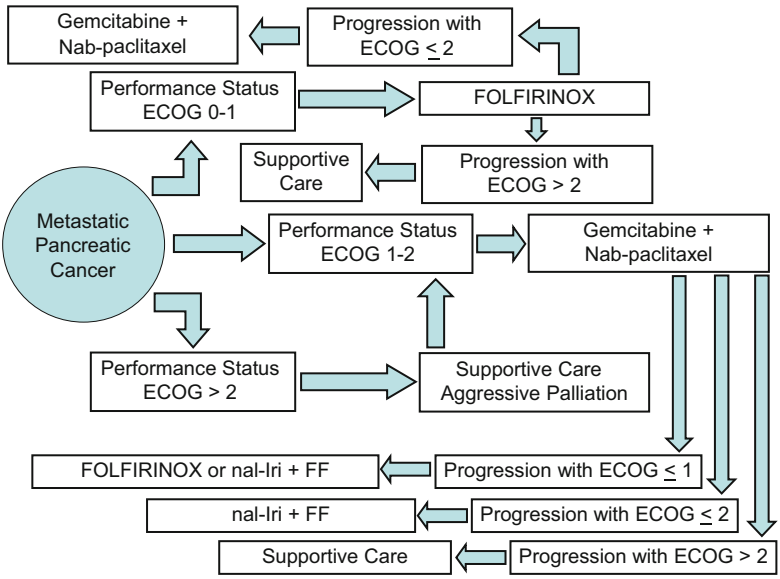

Fig. 4 Decision-analysis in patients with metastatic disease. Multiagent cytotoxic chemotherapy should be limited to those patients with good PS (ECOG 0-2) with distinctions for choosing gemcitabine/nab-paclitaxel vs FOLFIRINOX. Clinicians should be mindful of patients who have worsening PS during evaluation phase or when failing front line therapy. Supportive care may lead to improved PS increasing the potential benefit of cytotoxic therapy while decreasing its risks. Second line therapy is appropriate for those patients maintaining PS after progression on front line therapy

therapy. After the establishment of gemcitabine as standard treatment, there was some effort to establish a second-line regimen for patients who progressed on frontline gemcitabine-based therapy. Two distinct regimens of fluorouracil and oxaliplatin have been evaluated in randomized trials with conflicting results. CONKO-03 reported on a regimen of oxaliplatin, folinic acid, and fluorouracil (OFF) which is less dose intense than FOLFOX [65]. The study randomized 160 advanced pancreatic cancer patients with KPS $\geq$ 70%. Patients who randomized to OFF had a median overall survival of 26 weeks which was statistically and clinically significant in comparison to an overall median survival of 13 weeks for patients randomized to FF ($p = 0.014$). However in the PANCREOX trial conducted in Canada, there was no difference in PFS for the patients randomized to either modified FOLFOX6 (mFOLFOX6) or FF (3.1 months vs 2.9 months, $p = 0.99$) [66]. Of note, there was a difference in OS with patients randomized to FF having a median survival of 9.9 months versus 6.1 months for FOLFOX, $p = 0.02$. This difference was possibly explained by the higher use of third-line therapy among those patients randomized to FF compared with those randomized to FOLFOX (25% vs 6.8%, respectively).

FOLFIRI has also been investigated as a potential second-line therapy after initial gemcitabine-based treatment. Of note however, based on a small randomized trial conducted in patients with gemcitabine-refractory pancreatic cancer, there appears to

be no significant difference between second-line treatment with FOLFOX or with FOLFIRI [67].

Most recently, a large randomized trial reported on the benefits of nal-iri in combination with FF for patients previously treated in first-line with gemcitabine-based therapy. [60] The trial enrolled 417 patients with KPS $\geq$ 70% in a 1:1:1 randomization between nal-iri with FF, nal-iri monotherapy, or FF. Importantly, all patients had previous treatment with gemcitabine or a gemcitabine combination with approximately 30% of patients having prior therapy with a platinum analog. As previously described, the combination of FF and nal-irinotecan was superior to the other arms in terms of OS (6.1 months vs 4.9 months for nal-irinotecan or 4.2 months for FF; $p = 0.012$). Based on these results, nal-iri has been approved for use in patients who have failed frontline gemcitabine-based therapy.

Given the shift in systemic therapy to gemcitabine/nab-paclitaxel and FOLFIRINOX, it is currently uncertain what role nal-iri/FF will play in second-line therapy for patients with pancreatic cancer. For patients with KPS = 70% who have failed prior gemcitabine monotherapy or gemcitabine/nab-paclitaxel, therapy with FF/nal-iri appears reasonable. However, for patients who have failed gemcitabine-based frontline therapy and who maintain KPS $\geq$ 80%, whether to use FOLFIRINOX or FF/nal-iri is an open question. Lastly, it seems unlikely that patients who have previously failed FOLFIRINOX will benefit from FF/nal-iri, and in the community setting, gemcitabine/nab-paclitaxel is frequently used after front-line therapy with FOLFIRINOX. Support for this strategy comes from a retrospective analysis of 57 patients who received gemcitabine/nab-paclitaxel after FOLFIRINOX failure [68]. The objective response rate was 17.5% with a median OS from the start of second-line therapy of 8.8 months. Of note however, grade 3–4 toxicities were reported for 40% of these patients.

No matter what the initial frontline therapy, when clinicians decide on the merits of any second-line therapy, it may be worth considering a retrospective analysis from the University of Heidelberg [69]. The progression-free and overall survival of 46 patients who progressed after receiving palliative therapy at that institution was tracked. Patients with time to progression (TTP) less than 6 months on frontline therapy (TTP1) had a TTP on second-line therapy (TTP2) of only 2.2 months and a residual survival of 4.4 months. However, for patients with TTP1 >6 months, the residual overall survival was 7.5 months with second-line therapy. Although this finding has not been confirmed in a prospective trial, it still may influence a patient or clinician's enthusiasm for second-line therapy.

Surgery or Other Noninvasive Ablative Strategies May Be Relevant for a Small Subset of Stage IV Patients with Limited Metastatic and/or Persistent Local Disease After Initial Systemic Therapy

With wider use of FOLFIRINOX and gemcitabine/nab-paclitaxel, some dramatic responses are being reported in patients with pancreatic cancer [70, 71]. When such results are coupled with an expanding array of radiation options and noninvasive

ablative techniques, discussions about the merits of other aggressive interventions (to include surgery) are beginning to emerge [72, 73]. At the present time, there is no data to support or repudiate efforts to enhance systemic therapy with radiation, ablative strategies, or even surgical resection for limited metastatic disease or the primary tumor. However, some guiding principles may allow for a disciplined approach to decisions about interventions beyond systemic therapy for patients who present with metastatic disease [74].

The Ultimate Decision: Withholding or Terminating Anticancer Therapy

It is not particularly uncommon for patients with advanced pancreatic cancer to die during active anticancer therapy related to the underlying malignancy more so than toxicity. In an analysis of GI cancer patients treated on randomized trials at the Royal Marsden Hospital from 1992 to 2001, the 60-day all-cause mortality among 171 advanced pancreatic cancer patients was 13% [75]. Almost all of these deaths were attributed to the cancer itself with very few related to treatment toxicity. Nevertheless, the majority of pancreatic cancer patients will have to confront the decision to withhold or terminate cancer-directed therapy prior to their death. Most oncologists recognize that as a patient's condition declines or as proven therapies are exhausted, the risk of further cytotoxic therapy begins to outweigh its potential benefits. Importantly though, analysis of Medicare beneficiaries reveals that although the use of hospice services is increasing over time, the proportion of patients with pancreatic cancer who receive chemotherapy within the last month of life is also rising [76].

With this in mind, it is important for oncologists to communicate goals of care openly and early in the patient's disease course. With rare exception, patients with advanced disease should be informed on initial consultation that therapy will not be curative but the palliative benefits may be significant. Moreover, they should be told that if a particular therapy leads to tumor control or regression, it will be finite in its duration and that eventually, subsequent cancer-directed therapies will be ineffective. It should also be acknowledged that disease progression will ultimately lead to a decline in performance status making the delivery of further cytotoxic therapy not only futile, but possibly detrimental. Importantly, patients should be assured that even when active therapy is not recommended at all, or when discontinuation is advised, the patient's care needs will continue to be met. Lastly, while not comfortable for many physicians, a willingness to discuss spirituality and how spiritual beliefs may impact decisions about cancer-directed treatment or life-sustaining interventions may be both informative and gratifying.

Conclusion

Clinical decisions in pancreatic cancer can be challenging, particularly given the physical and emotional distress associated with this disease. Selection of patients for initial surgery requires high-quality staging studies, a careful evaluation of the patient's

potential for recovery, a full understanding of the current evidence, and a minimal amount of emotion. This will identify the patients most apt to benefit from surgical intervention and adjuvant therapy. For patients with BR pancreatic cancer, neoadjuvant therapy is the preferred initial intervention based on NCCN guidelines, but this approach is not accepted worldwide. In LAPC, systemic therapy is the standard of care. However, for the subset of patients who remain with local disease only, stable or progressing, SBRT and other novel ablative techniques are being investigated. Furthermore, up to 25% of patients with LAPC may be eligible for surgical resection with curative intent after aggressive multimodal therapy. Lastly, for those patients who present with metastatic disease, careful assessment of PS is critical to decision-making in regard to the utility of systemic therapy, how aggressive it should be, and whether second-line therapy is appropriate. For all patients, thoughtful clinical decision-making is a critical ingredient of compassionate cancer care.

Cross-References

- ▶ Adjuvant Chemoradiation Therapy for Pancreatic Cancer
- ▶ Adjuvant Chemotherapy in Pancreatic Cancer
- ▶ Diagnostic Biomarkers
- ▶ Interventional Radiology for Pancreatic Cancer
- ▶ Laparoscopic Staging in Patients with Newly Diagnosed Pancreatic Cancer
- ▶ MRI and MRCP for Diagnosis and Staging of Pancreatic Cancer
- ▶ Neoadjuvant Chemotherapy in Pancreatic Cancer
- ▶ New Japanese Classification of Pancreatic Cancer
- ▶ Palliative Surgery in Advanced Pancreatic Cancer
- ▶ Pancreatic Adenocarcinoma: CT and PET/CT
- ▶ Secondary Screening for Inherited Pancreatic Ductal Adenocarcinoma
- ▶ Venous Resection in Pancreatic Cancer Surgery

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Paraneoplastic Syndromes in Pancreatic Cancer

Jens Werner and Stephan Herzig

Contents

Abstract

Paraneoplastic syndromes are defined as signs and symptoms which present distant to the site of primary cancer or metastases. However, they are closely associated with the malignant disease and comprise metabolic, dystrophic, and/or degenerative symptoms, which are consequences of humoral or hormonal factors. The clinical symptoms vary widely and include systemic and organ-specific manifestations. In some cases, these can become the major clinical problems

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determining survival. Systemic manifestations include frequent symptoms of pancreatic cancer patients such as fever and cachexia. Organ-specific symptoms may represent as cutaneous, neurological, hematological, or endocrine symptoms. A special focus of this chapter is on diabetes mellitus associated with pancreatic tumors. The best-understood syndromes result from tumor production of biologically active substances or, to a lesser extent, from autoimmune phenomena. Biological active agents may promote the growth of the tumor directly. In turn, growth-promoting agents of this type may become the focus of new approaches to anticancer treatment. After successful treatment of the underlying malignant disease, paraneoplastic symptoms may resolve completely. Thus, early recognition of paraneoplastic syndromes is very important in the management of patients with pancreatic cancer. In the following chapter, the most common paraneoplastic syndromes are described in detail.

Keywords

Paraneoplastic syndrome · Systemic manifestation · Organ-specific manifestation · Diagnostic value · Treatment options · Monitoring of disease progression · Diabetes mellitus · Fever · Cachexia · Cutaneous manifestation · Neurological manifestation · Hematologic symptoms · Pancreatic enzymes and metabolism

Introduction

In most cases, pancreatic tumors produce clinical symptoms as a result of local expansion, with obliteration of normal tissues, as the malignant cells proliferate within the confines of the involved organ. Subsequently, the tumor compresses and infiltrates blood vessels, lymphatics, and nerve fibers as well as surrounding organs. Thus, the principal clinical presentation of pancreatic carcinoma includes abdominal pain and jaundice. While endocrine tumors may present with typical signs and symptoms as a consequence of the overproduction of specific hormones, benign and cystic tumors of the pancreas are mainly detected on routine radiographic evaluations in asymptomatic patients.

Paraneoplastic syndromes are defined as signs and symptoms which present distant to the site of primary cancer or metastases. However, they are closely associated with the malignant disease and comprise metabolic, dystrophic, and/or degenerative symptoms, which are consequences of humoral or hormonal factors. The clinical symptoms vary widely and include systemic and organ-specific manifestations. In some cases, these can become the major clinical problem and determine survival. Systemic manifestations include frequent symptoms of pancreatic cancer patients such as fever and cachexia. Organ-specific symptoms may present as cutaneous, neurological, hematological, or endocrine symptoms. The best-understood syndromes result from tumor production of biologically active substances or, to a lesser extent, from autoimmune phenomena. These would appear

to be probable mechanisms in many recognized paraneoplastic syndromes of uncertain etiology and perhaps in some unrecognized paraneoplastic syndromes.

The incidence of paraneoplastic syndromes is more frequent than generally suspected. Syndromes may occasionally be helpful in the diagnosis of cancer or in monitoring response to cancer therapy. They may produce symptoms as a result of their intrinsic biological activity. Biologically active agents produced by malignant cells may serve as markers early in the course of the disease and may increase the chance of early recognition and subsequent cure. In some patients, amelioration of the syndromes can reverse the patient's dominant symptoms and thus provide significant clinical palliation. Biological active agents may promote the growth of the tumor directly. In turn, growth-promoting agents of this type may become the focus of new approaches to anticancer treatment. After successful treatment of the underlying malignant disease, paraneoplastic symptoms may resolve completely. Thus, early recognition of paraneoplastic syndromes is very important in the management of patients with pancreatic cancer. In the following chapter, the most common paraneoplastic syndromes are described in detail.

Classical Symptoms of Pancreatic Cancer

The classical symptoms of pancreatic cancer include abdominal pain, jaundice, or an episode of acute pancreatitis.

Abdominal pain present in two-thirds of patients with pancreatic cancer [1]. Pain has usually been present to some degree for 2–3 months before presentation to the primary physician and is mainly a constant pain located in the epigastric region. Back pain may also be observed and seems to be a consequence of retroperitoneal infiltration of the plexus and nerves in large tumors which are often located in the corpus and tail of the pancreas. However, even pain may be a paraneoplastic symptom.

Jaundice is an early symptom of pancreatic head cancers and occurs in almost half of all patients with pancreatic tumors secondary to bile duct obstruction. Painless jaundice is typical as patients rarely present with biliary colics. Nevertheless, most of the patients have pain to a certain degree. While most cancers of patients with jaundice are located in the head of the pancreas, some cancers might be located in the distal portion of the pancreas and obstruct the bile duct by metastasis of the periportal lymph nodes.

A small number of patients with pancreatic cancer will present with an initial episode of acute pancreatitis. This is mostly the consequence of pancreatic duct obstruction by cancer or mucin derived from main duct IPMN's. Thus, especially in elderly patients without typical risk factors for acute pancreatitis, pancreatic cancer should be ruled out by additional diagnostics in those patients.

However, the described "typical symptoms" are not specific enough to allow the clinician to make a confident diagnosis of pancreatic cancer without an additional laboratory, radiological, or pathological examinations.

Endocrine tumors of the pancreas often present with specific symptoms as a consequence of an overproduction of organ-specific hormones. The different types of endocrine pancreatic tumors and their syndromes are described in detail in this chapter (endocrine paraneoplastic syndromes), as well as in chapters 21 and 44 of this book.

Clinical Manifestation and Diagnostics of Paraneoplastic Syndromes

The clinical symptoms of paraneoplastic syndromes vary widely and include systemic and organ-specific manifestations.

Systemic Manifestation

Cachexia

One of the major and most characteristic problems observed in cancer patients is weight loss, usually associated with anorexia. Compared to other tumors, pancreatic cancer has the highest incidence of cachexia reaching as much as 80% of all patients at the time of diagnosis [2]. As a consequence, palliation of this occurrence remains one of the most important therapeutic targets in clinical practice. Over the past years, important new developments regarding the pathogenesis of pancreatic cancer associated cachexia have been achieved.

Anorexia represents the failure of usual appetite signals whereas cachexia is the debilitating state of involuntary weight loss. This syndrome is defined as the "cancer anorexia-cachexia syndrome" [3]. This syndrome usually consists of a combination of anorexia, tissue wasting, malnutrition, weight loss, abnormalities of taste and smell, and the impossibility to increase oral intake to adapt energy expenditure. The cause for this commonly observed and often life-limiting disturbance remains to be determined in spite of the fact that many contributing factors have been identified. The pathogenesis is multifactorial.

The patients often simply can not ingest food, despite the need for increased nourishment. An aversion to meat and nausea is often observed. Early satiety is probably also the consequence of gastroparesis, delayed gastric emptying, and postprandial bloating. Malignant gastroparesis may result from cancer itself or may be a complication of its treatment including surgery, radiotherapy, or chemotherapy [4]. Potential pathophysiological mechanisms of malignant gastroparesis include postvagotomy syndrome, malignant infiltration of the autonomic nervous system, and paraneoplastic dysmotility with autoantibody-mediated destruction of the enteric nervous system. In addition, the loss of appetite and weight is a consequence of abdominal pain, restricted food intake due to duodenal stenosis, and maldigestion secondary to exocrine insufficiency.

Specific disorders of carbohydrate, protein, lipid, and energy metabolism also play a crucial role in the pathophysiology of the catabolic state observed in most

patients with pancreatic cancer. Biochemical abnormalities in energy metabolism have been well characterized. Fatty acids are oxidized in preference to glucose, and anaerobic glucose metabolism is increased while oxidative phosphorylation is reduced. This results in an inefficient expenditure of ATP, and a subsequent energy deficit. A complex network of cytokines, neuroendocrine hormones, and tumor-derived factors seem to further mediate the catabolic changes [5]. Increase of proinflammatory cytokines including IL-1, IL-6, TNF-a, TGF-B, and others initiate the release of leptin, a hormone that is secreted by adipose tissue, and which is responsible for the homeostasis of body weight via a central negative feedback mechanism. High levels of leptin reduce hypothalamic orexigeneic mediators (e.g., orexin, ghrelin, neuropeptide Y) and increase anorexigenic mediators including thyroid-releasing hormone, glucagon-like peptide. As a consequence, a continuous increase of energy expenditure is induced [6].

Treatment of cachexia today includes symptomatic administration of hypercaloric parenteral and/or enteral nutrition. However, these management approaches have not been proven to be beneficial to improve symptoms or survival in pancreatic cancer patients [7]. Today, next to the application of progestogens (e.g., megestrol acetate) and corticosteroids, several experimental approaches such as inhibitors of pro-inflammatory cytokines are presently under investigation [8]. Thalidomide, an inhibitor of tumor necrosis factor-alpha, has recently been shown to stop weight loss in patients with cachexia and pancreatic cancer [9].

Fever

Fever is another frequent systemic sign which can be observed in about 10% of all patients with malignancy. However, infections including infections by endogenous bacteria or fungi need to be ruled out first, before it is considered to be paraneoplastic. The pathophysiological mechanisms of fever as a paraneoplastic symptom include increased cytokine release by cancer cells or immunoreactions of the tumor with subsequent IL-1 release by monocytes.

Other systemic paraneoplastic symptoms include arthritis, digital necrosis, or lactate acidosis. However, these syndromes are rarely observed in patients with pancreatic cancer.

Cutaneous Manifestation

Paraneoplastic dermatoses are markers of internal malignancy, characterized by being relatively uncommon, associated with certain forms of cancer and occurring in connection with cancer either before, during, or after the diagnosis has been made. Furthermore, the skin symptoms typically run a parallel course with the cancer. Most paraneoplastic dermatoses disappear when the primary tumour is removed and reappear in the case of recurrence or metastases of the cancer. Adult dermatomyositis is especially associated with breast and lung cancer. Cutaneous manifestations of patients with pancreatic cancer are very rare. Although larger series have not been reported, almost all kind of cutaneous paraneoplastic lesions have been described in

association with pancreatic tumors in case reports. Subsequently, the most frequent cutaneous manifestations are described.

Necrolytic Migratory Erythema

Necrolytic migratory erythema is a cutaneous paraneoplastic manifestation, which is usually associated with a glucagon-secreting pancreatic tumor (alpha-2 cell carcinomas of the pancreas). Although it also may occur in other circumstances in which serum glucagon is elevated, as in hepatic cirrhosis, it is more specific than all other cutaneous paraneoplasias for a certain tumor entity, the glucagon-secreting pancreatic tumors. Glucagonoma syndrome is a paraneoplastic phenomenon characterized by an islet alpha-cell pancreatic tumor, necrolytic migratory erythema, diabetes mellitus, weight loss, anemia, stomatitis, thromboembolism, and gastrointestinal and neuropsychiatric disturbances. These clinical findings in association with hyperglucagonemia and demonstrable pancreatic tumor establish the diagnosis. Glucagon itself is responsible for most of the observed signs and symptoms, and its induction of hypoaminoacidemia is thought to lead to necrolytic migratory erythema [10]. Necrolytic migratory erythema is characterized by a figurative eruption with erosions and a rapid centrifugal progression, that become necrotic and hyperpigmentated after healing. They are mainly located at groin, axillae, but can manifest everywhere (Fig. 1a, b).

Erythema Nodosum

Erythema nodosum is defined as painful subcutaneous nodules mainly located on the anterior surfaces of the legs. Physical examination reveals numerous firm, tender, erythematous and violaceous, subcutaneous nodules on the lower extremities, with marked bilateral pitting edema, and characteristic changes of fat necrosis [11] (Fig. 2a, b). Erythema nodosum may be observed in any pancreatic cancer, but is most common for acinar cell carcinoma. A concentration can be detected in the fluids from the cutaneous lesion which cause the subcutaneous inflammation and necrosis.

Acanthosis Nigricans

Acanthosis nigricans represents a localized hyperpigmentation with a velvety surface most often located in the neck, axillae, and groin, and occasionally on the

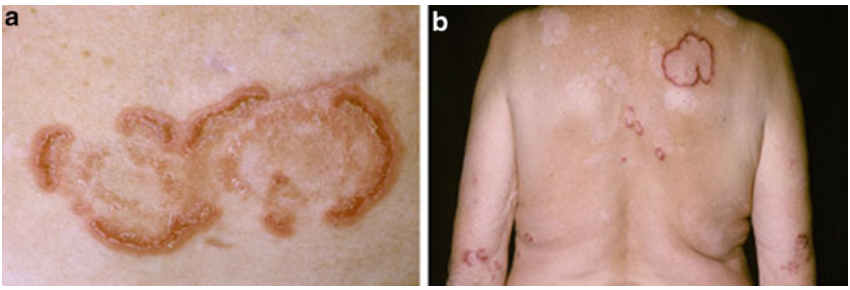

Fig. 1 (**a** and **b**) Necrolytic migratory erythema is characterized by a figurative eruption with erosions and a rapid centrifugal progression

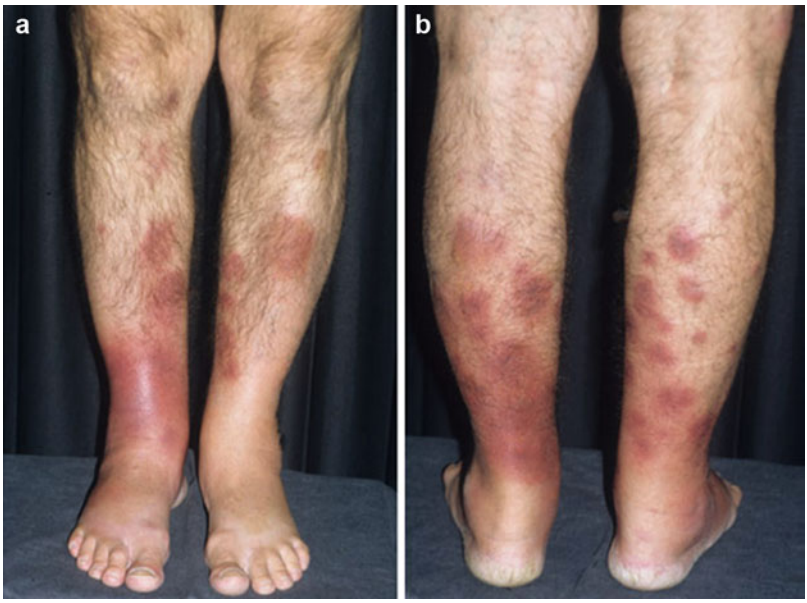

Fig. 2 (**a** and **b**) Erythema nodosum is characterized by subcutaneous nodules on the surfaces of the legs

dorsum of the hand and corners of the mouth. The malignant form is characterized by a rapid progression and pruritus. It is almost always associated with malignancies and therefore cancer search should be initiated once the diagnosis is established [12].

Leser-Trelaut

The Leser-Trelaut syndrome is defined by rapid development of multiple seborrheic lesions, which are often associated with skin tags and acanthosis nigricans. They are mostly located on the back, although it can manifest everywhere.

Akrokeratosis Paraneoplastica

Bazex syndrome is a rare cutaneous paraneoplastic phenomenon which is normally associated with cancers of the upper respiratory tract and digestive tract, which has also been reported in association with pancreatic neuroendocrine tumors. It may be treated successfully with octreotide. It is mainly located in the nose, ears, or fingertips, and has to be distinguished from psoriasis and Lupus.

Eczematous Dermatitis

Skin diseases may be the presenting sign of malignancy, but strict criteria are required to make the diagnosis of a paraneoplastic syndrome. Common dermatoses may also be associated with an underlying malignancy. Thus, in case of unresponsive eczematous dermatitis, an underlying malignant disease, including pancreatic cancer, should be considered in certain cases. This is especially true, if

the lesions behave in an atypical or aggressive manner or are not successfully treated by standard therapies.

Neurological Manifestation

Neoplasms can derange neurologic function in a number of ways, including direct invasion, metastatic invasion, by opportunistic infections, as complications of antineoplastic treatment, metabolic complications, or paraneoplastic syndromes. Depression is a frequent manifestation of advanced cancer and may be an initial symptom of some patients with pancreatic cancer [13]. Depression may be a specific biological attribute of the disease or, since it is mainly diagnosed at a late stage, a secondary manifestation of a life-threatening illness.

Paraneoplastic neurological syndromes present heterogeneous. Nevertheless, they share several characteristics. Paraneoplastic neurological manifestations are clinically dramatic and arise subacutely within several weeks or even days to produce neurologic symptoms that may be very disabling. The syndromes may precede the detection of the malignancy by months. Similarly to the cutaneous manifestations, almost every symptom and even more than one syndrome may be induced by a certain neoplasm. However, certain clinical manifestations are associated with particular types of tumors in the majority of cases [14]. Pancreatic tumors are rarely associated with neurological paraneoplastic syndromes. While paraneoplastic neurological syndromes of the brain and cerebellum and the spinal cord are not associated with pancreatic neoplasms, polyneuropathies and neuromuscular paraneoplasias such as myasthenia gravis and pseudomyasthenia have been reported to be associated with pancreatic neoplasms in some cases. Autoimmune mechanisms have been implicated in these paraneoplastic disorders, which are characterized by highly specific patterns of reactivity with neural tissue or muscle. In both, the myasthenia gravis and the pseudomyasthenia syndrome, circulating antibodies that are directed at synaptic proteins have been identified. In addition, both diseases have been reproduced in animals by passive administration of fractionated immunoglobulins.

Neuromuscular Paraneoplastic Syndromes

Myasthenia Gravis

Fifteen percent of the cases are associated with thymoma. Rarely, other tumors including pancreatic neoplasias are the underlying cause. Myasthenia gravis is characterized by exercise-induced muscle weakness caused by antibody-mediated reduction in the number of acetylcholine receptors at the postsynaptic junction.

Pseudomyasthenia (Lambert-Eaton.Syndrome)

This syndrome is characterized by weakness, myalgias, and fatigue, typically pronounced in the lower extremities and proximal muscles. The incidence of underlying malignant disease is about 70%. Typically, there is a striking reduction of strength in

rest and a transient improvement of energy on repetitive exercise. Common associated features are dryness of mouth and eyes, diminished sweating, and orthostatic symptoms. The syndrome is believed to be an autoimmune disorder with diminished release of acetylcholine and a decreased number of active zones in the presynaptic terminal.

Dermatomyositis, Polymyositis

Up to 3% of patients with dermatomyositis or polymyositis have a malignant underlying disease, which mostly is diagnosed within 1 year after the first symptoms [15]. Malignant disease has to be considered especially in older patients with myositis. The main clinical symptom is a progressive weakness of the proximal muscles, with muscle enzymes being increased. The EMG is pathological and biopsies reveal necrosis and a mild inflammation. The cause of this paraneoplastic syndrome is either an altered immune status or an occult viral infection.

Polyneuropathies

Paraneoplastic polyneuropathies are the most frequent tumor-associated neurological diseases. They are observed with almost any tumor, although lung carcinoma and ovarian carcinoma are the most commonly observed entities. The clinical symptoms are unspecific and cannot be distinguished from neuropathies of other etiologies. The symptoms include atrophy of muscles, distal hypoesthesia, and pain. A treatment is not known, but symptoms improve after management of the underlying malignant disease.

Hematologic Manifestation

Disorders of all three cell lines of the hematopoetic system and the coagulation cascade are frequently observed in carcinoma patients (about 5% of all cancer patients). The most frequent cause is the infiltration of the bone marrow, infectious or toxic complications.

Anemia is found with increased incidence in advanced tumor stages of malignant diseases. The etiology of anemia cannot be determined in most of the cases. In addition, there is no association of anemia with a special type of carcinoma. In general, the mechanisms accounting for anemia are almost exclusively extrinsic to the tumor and include the destruction of erythrocytes from hypersplenism, microangiopathic hemolysis, and autoantibodies, as well as anemia secondary to gastrointestinal bleeding and many other circumstances.

Microcystic pancreatic tumors are reported to be associated with an autoimmune-induced hemolytical anemia, and patients with mucin-producing adenocarcinomas of the pancreas have been reported to develop microangiopathic-induced hemolytic anemia. Pathogenetically, tumor cell invasion induce endothelial lesions and disturbances of the microcirculation with subsequent fragmentation of erythrocytes. A thrombotic-thrombocytopenic purpura or a hemolytic-uremic syndrome, and finally a clinical disseminated coagulopathy (DIC) may develop.

Pancreatic tumors associated with **polycythemia** have not been described.

Similarly, a **leukocytopenia** has not been reported as a paraneoplastic syndrome in patients with pancreatic cancer.

In contrast, **leukocytosis** is frequently found in different malignant diseases including pancreatic carcinomas. The pathophysiological mechanism is a cytokine-mediated increase of growth-factors (e.g., G-CSF, GM-CSF). In general, leukocytosis is asymptomatic, but leukemoid reactions have been described in pancreatic cancer [16]. An **eosinophilia** has been associated with several malignancies and also with pancreatic cancers. Fever, allergic reactions, and an eosinophilic pulmonary infiltration might be observed in these patients. Another rare disease is the **Sweet-syndrome,** which is associated with malignancies including pancreatic cancers in about 20%. It is characterized by an acute febrile dermatosis which is associated with arthralgia, myalgia, pulmonary infiltrations, and glomerulonephritis.

Thrombocytosis is frequently associated with almost any malignant disease, but is rarely of any pathological significance. Symptomatic thrombosis and hemorrhagias are rarely observed. **Thrombocytopenia** is not associated with patients with pancreatic cancer.

However, pancreatic cancer patients and patients with mucin-producing tumors and adenocarcinomas of the pancreas are frequently (about 18%) associated with a hypercoagulable state and a clinical **disseminated coagulopathy**. Consequently, venous thrombosis and pulmonary embolism, as well as nonbacterial endocarditis develop quite frequently. Pathophysiological mechanisms are increased thromboplastin levels which are increased in tumor compared to normal tissue and a direct activation of factor X by tumor-derived procoagulatory factors.

Endocrine Manifestation

The term “ectopic hormone secretion” defined tumor-derived hormone production of tissues which normally do not release any hormones. Although this term is still used today, it is well known that many human tissues apart from the typical endocrine tissues produce hormones. The following criteria should be fulfilled to define “ectopic hormone production”:

- Reduction of hormone level and decrease of paraneoplastic symptoms after removal of tumor
- Persistence of increased hormone levels after resection of the organ which normally produces the hormone
- Identification of an artero-venous difference of hormone concentrations in the vascular system of the tumor
- Detection of hormones in the tumor tissue and production of hormones in the in vitro cultures of the tumor tissue

Pathogenetic cause of the hormone production is the genetic depression and genetic mutations during tumorigenesis [17].

Pancreatic tumors may be associated with the following paraneoplastic endocrine syndromes:

- **Acromegaly**: The increased production of Growth hormone–releasing hormone (GHRH) and growth hormone (GH) in the absence of a pituitary adenoma. Most of these pancreatic tumors are located in the tail of the pancreas. The clinical manifestation is identical to acromegaly.
- **Syndrome of inadequate ADH-secretion** (Vasoactive intestinal peptide): Schwartz and Bartter described the first cases in 1957 which presented with symptoms including hyponatremia, hypervolemia, and increased urine osmolality. The syndrome is caused by an increased level of vasopressin, which may be the consequence of increased ADH or ANP production. Clinical symptoms may develop and include headache, nausea, vomiting, disorientation, and convulsions.

Diabetes Mellitus

Classification of Diabetes Mellitus

Diabetes mellitus comprises a group of heterogeneous metabolic disorders, which has an increase in blood glucose levels in common. Whereas the so-called type I diabetes results from the autoimmune destruction of pancreatic beta cells, type II diabetes is caused by the insensitivity of peripheral organs such as muscle, fat, and liver against the action of the pancreatic peptide hormone insulin (i.e., insulin resistance), combined with an inability of the beta cell to respond normally to glucose by appropriately increasing insulin secretion [18]. Thereby, type II diabetes accounts for more than 90% of diabetes worldwide [19]. While the relative contribution of these two defects to type 2 diabetic pathogenesis is still under debate, longitudinal studies in high-risk individuals suggest that insulin resistance is an early phenomenon, occurring many years before any signs of glucose intolerance, whereas the beta cell failure develops later in the pathogenesis of disease [20]. Both hallmarks of type 2 diabetes, insulin resistance and beta cell failure, seem to arise from a complex interplay between different genetic and environmental pathways and factors. In this regard, estimates suggest that 30–70% of type 2 diabetes risk can be attributed to genetic factors in a polygenic and heterogeneous manner [21]. This indicates that a variety of distinct genes and different genetic combinations are involved in type 2 diabetic pathogenesis, which in turn intertwine with a number of environmental conditions and risk factors (e.g., high-caloric food intake, life style, aging) [22].

Apart from type 1 and type 2 diabetes, there are additional specific types of diabetes including paraneoplastic, maturity onset, or gestational diabetes. Particularly exo- and endocrine tumors of the pancreas display a high prevalence of diabetes mellitus as a paraneoplastic syndrome, most likely reflecting the close interrelationship between exocrine and endocrine cells within the pancreas and their importance for overall energy homeostasis under nonneoplastic conditions [23].

In clinical terms, several criteria may be used to establish the diagnosis of diabetes. (A) a 75 g oral glucose tolerance test with a 2 hour value of 200 mg/dL or more, (B) a random plasma glucose of 200 mg/dL or more with typical symptoms

of diabetes, or (C) a fasting plasma glucose of 126 mg/dL or more on more than one occasion [7]. In most cases, fasting glucose values are preferred for their convenience, reproducibility, and correlation with increased risk of microvascular complications. In this context, impaired fasting glucose has been defined as fasting plasma glucose of 110 or more but less than 125 mg/dL on two different days. In addition, impaired glucose tolerance is defined as a plasma glucose value of 140 or more along with less than 200 mg/dL during an oral glucose tolerance test 2 hours after [24].

Control of Metabolism in Health and Diabetes through Pancreatic Hormones

Under normal, nonneoplastic conditions, the pancreatic beta cell hormone insulin triggers the fast uptake and oxidative catabolism of glucose in liver, muscle, and adipose tissue, and simultaneously inhibits glycogenolysis and gluconeogenesis in liver during feeding [25, 26].

All of the actions of insulin are mediated by its membrane-bound receptor, a member of the tyrosine kinase receptor family [27]. Upon insulin binding, the intrinsic tyrosine kinase activity of the insulin receptor at the cell surface becomes activated and leads to the subsequent tyrosine phosphorylation of multiple signaling components, involving phosphoinositide-3-kinase and the Ser/Thr kinase protein kinase (PK) B/Akt, thereby transducing the insulin signal to downstream cytoplasmic and nuclear effectors which then ultimately control insulin's metabolic effects [27–32].

In particular, insulin signaling results in translocation of glucose transporter 4 from its intracellular pool to the plasma membrane and glucose transport into skeletal muscle and adipose tissue [33, 34], thereby effectively lowering circulating blood glucose levels. In adipose tissue, insulin acts also antilipolytic, whereby it inhibits the release of fatty acids from adipocytes by decreasing the activity of hormone-sensitive lipase and adipose triglyceride lipase (ATGL). In the liver, insulin prevents the release of glucose from the liver by inhibiting hepatic glycogen breakdown to glucose and the expression/activity of key enzymes in the de novo glucose production pathway (i.e., gluconeogenesis) [35, 36]. The importance of functional insulin signaling for whole-body survival and homeostasis can be most dramatically demonstrated in mouse models of total body deficiency in insulin receptor expression, leading to severe ketoacidosis and death of the affected animals shortly after birth [37].

Low plasma glucose levels during fasting and exercise trigger a series of hormonal cues that promote a switch in whole body energy usage. Along with a drop in insulin levels, counter-regulatory hormones gain metabolic control. In particular, the peptide hormone glucagon from alpha cells within the pancreatic islets and adrenal glucocorticoids are released into the circulation [38–41]. These hormones activate triglyceride breakdown via the induction of hormone-sensitive lipase in white adipose tissue and contribute to glycogen degradation in both muscle and liver, thereby leading to the release of previously stored glucose depots and an elevation of blood glucose concentrations [26].

In addition, the high availability of circulating, adipose tissue-derived lipids determines the enhanced mitochondrial oxidation of free fatty acids (FFA) in the

liver. The oxidation end product, acetyl-CoA, serves as a substrate for the synthesis of ketone bodies that are exported from the liver and used as primary energy source by skeletal muscle or brain after prolonged starvation periods. Apart from providing acetyl-CoA, FFA beta-oxidation represents a critical energy provider for hepatic gluconeogenesis. The gluconeogenic pathway represents a prominent feature of liver metabolism and acts as the primary defense mechanism against hypoglycemic conditions in response to glucagon/glucocorticoid signaling during fasting through the provision of glucose for extrahepatic tissues such as erythrocytes, renal medulla, and brain [42–45].

The execution of gluconeogenesis and FFA oxidation during fasting and the consequent provision of energy substrates are supported by the concomitant inhibition of insulin-dependent anabolic pathways. In this regard, under the influence of glucagon and glucocorticoids, mitochondrial FFA utilization is promoted by the simultaneous repression of insulin-dependent hepatic lipid storage and synthesis (lipogenesis), and end-products of FFA oxidation, acetyl-CoA, and NADH, serve as allosteric inhibitors of insulin-dependent glycolytic enzymes, isocitrate dehydrogenase, and pyruvate dehydrogenase [26, 46–50]. On the other hand, insulin efficiently and actively blocks counter-regulatory gluconeogenic and beta-oxidation pathways to ensure appropriate energy storage in the fed state [51, 52].

Endocrine regulatory circuits of pancreatic islet peptide hormones, thereby represent critical checkpoints in the overall metabolic adaptation of glucose and energy homeostasis in response to dietary or environmental challenges.

Consequently, either loss or impairment of insulin signaling, insensitivity against its action (i.e., insulin resistance), or a nonphysiological dominance of counter-regulatory hormones, particularly glucagon, results in severe metabolic dysfunctions such as hyperglycemia and dyslipidemia, ultimately leading to the manifestation of diabetes mellitus.

In this context, relative or absolute insulin deficiency and/or elevated glucagon action are causative for decreased insulin-dependent glucose uptake into skeletal muscle and adipose tissue, derepression/activation of hepatic glucose production in the liver, and increased lipolysis in adipose tissue, leading to systemic hyperglycemia and dyslipidemia. Indeed, even a dysfunctional metabolic response of an individual tissue to imbalances in hormone levels can cause severe systemic pathologies. To this end, a defective insulin response in the liver has been shown to importantly contribute to the development of overall peripheral insulin resistance [53–55]. Mice bearing a targeted disruption of the insulin receptor gene in liver display hyperglycemia, hyperinsulinemia, and impaired glucose tolerance [56]. Also, inhibition of the PI3K/Akt-dependent insulin signaling pathway in the liver by the Akt-inhibitor TRB3 leads to hyperglycemia and glucose intolerance [57]. In contrast, reconstitution of insulin signaling by transgenic expression of a constitutively active insulin receptor specifically in liver reverses hyperglycemia and improves glucose tolerance as well as survival of insulin receptor deficient mice [58].

Interestingly, in addition to the manifestation of endocrine pancreatic tumors which can directly disrupt physiologic hormone balance and levels (see below),

many lines of evidence have shown that chronic activation of proinflammatory pathways within insulin target cells can lead to impairment of insulin signaling and diabetes.

Indeed, in addition to classical acute inflammation, metabolic diseases, such as obesity, atherosclerosis, and cancer, have been recognized as low-grade, subacute inflammatory conditions, contributing to the development of end-stage diseases such as diabetes [59, 60]. All of these conditions are characterized by elevated levels of proinflammatory cytokines, such as tumor necrosis factor (TNF) alpha, interleukins (IL) 1beta and 6, and various chemokines [60–62].

Toward this end, TNF alpha, IL6, IL1beta as well as other cytokine levels are elevated in patients and mouse models of impaired insulin signaling [63–65]. In this respect, ablation of the TNF alpha gene or of its receptor renders mice resistant to the development of insulin resistance and associated metabolic disorders [66, 67]. And, a common polymorphism of the IL6 receptor gene interacts with energy intake and affects adipose tissue mass in humans [68], underlining the critical impact of pro-inflammatory cytokine signaling for metabolic diseases. Consistent with this, elevated levels of the proinflammatory cytokines TNF-α, IL-6, and C-reactive protein (CRP) have been shown in individuals with insulin resistance and diabetes [69, 70]. At the cellular level, the inhibitory effects of proinflammatory cytokines on insulin action are mostly mediated via phosphorylation of certain serine residues on insulin receptor substrate (IRS)-1, including Ser312 (Ser307 in the rodent IRS-1 protein), Ser636 (Ser632 in the rodent IRS-1 protein), and Ser1101. Specifically, phosphorylation of these serine residues impedes the normal association of IRS-1 with the insulin receptor, thereby impairing downstream propagation of insulin signaling [71, 72].

Ultimately, alterations of insulin and/or glucagon signaling strength are translated into the activation or repression of gene-regulatory proteins, the so-called transcription factors, which in turn determine the activity status of tissue-specific genetic programs and consequent changes in cellular metabolism.

Research over the past decades has identified key molecular mediators of pancreatic hormone actions.

The FoxO proteins belong to a subfamily of Forkhead transcription factors which all have the so-called “winged-helix” like DNA-binding structure in common. In mammals, three major insulin-regulated FoxO-family transcription factors have been identified so far: FoxO1 (FKHR), FoxO3a (FKHRL1), and FoxO4 (AFX). In addition to the N-terminal “winged-helix-domain,” these three FoxO-proteins share several structural and functional characteristics. All of them have a C-terminal transactivation domain, a nuclear localization signal (NLS), a nuclear exclusion sequence (NES), and three RxRxxS/T consensus sites for phosphorylation by PKB/Akt. Phosphorylation of FoxO-proteins in response to insulin by PKB results in nuclear exclusion and thereby transcriptional inactivation of these proteins [73, 74]. Indeed, in vitro studies have linked FoxO proteins with the transcriptional regulation of insulin-responsive genes involved in carbohydrate and lipid metabolism [75]. The expression of most of the genes, e.g., gluconeogenic phosphoenolpyruvate carboxykinase (PEPCK) and the glucose-6-phosphatase catalytic subunit

(G6Pase), is suppressed by insulin, and the inhibition of FoxO-activity by insulin-induced phosphorylation is regarded as the major mechanism for this regulation [76]. In agreement with this, the binding sites of FoxO proteins within these gene promoters have been characterized as insulin-responsive cis-regulatory DNA elements in this setting [77]. Interestingly, these sites were frequently characterized as insulin-responsive elements long before they were characterized as Foxo-binding sites. Systemically, the partial loss of Foxo1 function decreases hepatic glucose production and promotes adipogenesis and beta cell development [78, 79], processes critically involved in the maintenance of systemic energy homeostasis and glycemic control.

In addition to FoxO transcription factors, members of the nuclear receptor transcription factor family have been identified as major insulin-responsive regulatory factors, most notably the peroxisome proliferator-activated receptor (PPAR) γ. PPARγ is expressed in all major insulin-sensitive tissues, with highest levels in adipose tissue [80]; and its transcriptional activity has been causally linked to the maintenance of peripheral insulin sensitivity in humans [81, 82] as well as animal models [83–89]. Importantly, the antidiabetic action of insulin sensitizers of the thiazolidinedione (TZD) drug family is conferred through their ligand and activation function for PPARγ, establishing PPARγ as the major molecular target molecule in diabetes therapy [90–92].

Genetic Susceptibility to Diabetes

In combination with environmental, hormonal, and/or inflammatory factors, susceptibility for diabetes mellitus seems to be also determined by genetic factors and predispositions [93]. Indeed, the role of genetics in type 2 diabetes is indicated by the familial clustering of insulin sensitivity and secretion, the higher concordance rate of typ II diabetes in monozygotic versus dizygotic twins, and the high prevalence of type II diabetes in certain ethnic groups (e.g., Pima Indians or Mexican Americans) [94, 95].

To this end, recent genome-wide association studies have identified a number of chromosomal loci associated with an increased risk for the development of diabetes.

In this regard, the strongest association of diabetes risk was found so far for the TCF7L2 locus. Individuals homozygous for the high-risk allele have about a doubling of diabetes risk [96, 97]. TCF7L2 represents a nuclear receptor for beta-catenin, critically involved in cell proliferation, adipogenesis, and pancreatic islet development [98]. In addition to TCF7L2, several other loci have been found to be significantly associated with diabetes risk, including the zinc transporter SLC30A8 [99], the homeobox transcription factor HHEX/insulin-degrading enzyme (IDE)/kinesin interacting factor (KIF) 11 locus comprising at least three potential diabetes genes [100], and the CDK5 regulatory subunit-associated protein 1-like 1 gene [101, 102]. Notably, all of these gene products have been implicated in beta cell insulin secretion, pancreas development and insulin degradation, or insulin gene expression, respectively [93], again pointing toward a tight cross-talk between acute (hormones) and permanent (genetic variants) determinants in the control of diabetic hyperglycemia and systemic energy balance.

Consistent with this notion, genome-wide association studies confirmed the importance of nuclear receptor PPARγ (see above) for insulin sensitivity and glucose homeostasis also on the genetic level with an odds ratio of 1.14 ($p = 1.7 \times 10^{-6}$) [103–105]. Genetic association studies thereby supported the impact of a long-known Pro12-to-Ala (P12A) polymorphism in the PPARγ2 gene on diabetes susceptibility. Resistance to diabetes is associated with the minor (Ala12) allele and susceptibility with the major allele (Pro12), which has a prevalence of about 85% among nondiabetic individuals and 88% among diabetic subjects. The genetic variation occurs specifically in the PPARγ2 isoform of the gene which is specifically expressed in adipose tissue and targeted by insulin sensitizer of the thiazolidinedione drug family [82, 106].

As variations of the above-described loci are linked to the susceptibility for metabolic dysfunctions under conditions of impaired insulin signaling and/or increased hormonal counter-regulation (glucagon), it is tempting to speculate that certain genetic variants may also determine the severity and outcome of paraneoplastic diabetes in the context of pancreatic tumor growth.

Pancreatic Endocrine Tumors

During development, endocrine and exocrine cell types within the pancreas arise from common precursors in the foregut endoderm. Within human pancreatic islets, insulin-producing beta cells are centrally located, whereas the islet periphery is populated by alpha, delta, and PP cells, secreting glucagon, somatostatin, and pancreatic polypeptide, respectively [107].

As islet cells demonstrate hormone coexpression during embryonic development, it is believed that pancreatic endocrine tumors (PETs) originate from multipotent cells in the ductal epithelium that retain their ability to differentiate into the corresponding endocrine cell type [108]. PETs are rare neoplasms of the pancreas accounting for less than 5% of all primary pancreatic malignancies [109]. In general, the prognosis for PETs is superior to the one of the more common ductal adenocarcinoma, even in metastasizing cases [110, 111]. As tumors originating from endocrine cells within the pancreatic islets, clinical syndromes associated with these malignancies mostly reflect the impact of the hormone secreted by the respective tumor. Consistently, the so-called insulinomas, glucagonomas, and somatostatinomas are characterized by dysfunctional systemic glucose homeostasis and paraneoplastic diabetes [112, 113]. Collectively, these neoplasms are classified as functional PETs, whereas endocrine tumors not associated with a clinical syndrome are referred to as a nonfunctioning PET. In contrast to functional lesions, nonfunctional PETs lack signs of hormonal hypersecretion and most commonly occur as space-occupying lesions with obstructive jaundice, gastrointestinal obstruction, bleeding, or upper abdominal pain, often presenting with a slow growth rate and developing in the head of the pancreas [113]. In contrast to functional PETs, which can be diagnosed on the basis of clinical symptoms and elevated blood hormone levels, the diagnosis of nonfunctional PETs relies on imaging techniques and histopathologic features. In this respect, multidetector computed tomography of

the abdomen is the most widely applied method for assessing the local extent of PET expansion and the presence of metastases [111, 112].

Among all PETs, insulinomas arising from insulin-producing beta cells represent the most common type of endocrine neoplasms (roughly 30–40% of all PET cases) [114].

Malignant insulinomas invade locally and metastasize to regional lymph nodes and the liver. Outcome depends on the stage of the disease. Malignant insulinomas are generally solitary and larger than their benign counterparts. The presence or absence of liver metastases is a predictor of survival. Despite their malignant potential, the majority of insulinomas are benign (90%) and localized within the pancreatic parenchyma [114, 115].

Within the pancreas, insulinomas are equally distributed throughout the gland and only found at ectopic locations in a small percentage of cases (3%), with the duodenal mucosa being the most common site of ectopic insulinoma growth. Due to an unchecked insulin production and secretion, insulinomas consequently present with hypoglycemia, sometimes accompanied by confusion, behavioral changes, blurred vision, fatigue, seizures, coma, and even death [111].

In diagnostic terms, an insulinoma represents a rare cause of hypoglycemia, and it is therefore mandatory to ensure that hyperinsulinemia is secondary to endogenous insulin production. Particularly, C-peptide and sulphonylurea levels should be assessed as low C-peptide concentrations are indicative of exogenous insulin administration, whereas sulphonylureas produce glucose and C-peptide levels similar to those found with insulinomas [116–118]. The following parameters are diagnostic for insulinoma: blood glucose $\leq$2.5 mmol/l, insulin $\geq$6 μunits/ml, c-peptide $\geq$0.2 nmol/l, and a negative sulphonylurea screen, obtained during a supervised fast with blood assessment every 6 h [111].

Following a biochemical diagnosis, tumor localization is important to plan treatment options, particularly surgical resection as the treatment of choice. Procedures employed include enucleation, distal pancreatectomy, and pancreaticoduodenectomy. As the majority of these tumors are benign, enucleation of the lesion may be feasible when preoperative scans and intraoperative ultrasonography demonstrate that the tumor is separate from the pancreatic duct by 2–3 mm and surrounding vascular structures [119]. As stated above, insulinomas are equally distributed throughout the pancreas. Consequently, a blind resection would fail to remove the tumor in 50% of cases. Intensive pre- and intraoperative localization of biochemically confirmed tumor is, therefore, mandatory to ensure maximum surgical success [120].

As described above, glucagon is the main counter-regulatory hormone of insulin action, being responsible for the maintenance of blood glucose levels during fasting under non-neoplastic conditions. Tumors of the pancreatic alpha cells are rare, but they may cause an increase in glucagon levels, resulting in impaired systemic glucose regulation and diabetic hyperglycemia [111]. Along with diabetes, glucagonomas typically present with dermatitis, deep vein thrombosis, and depression, commonly referred to as the 4 “Ds” [121, 122]. The pathognomic rash is known as necrolytic migratory erythema and may appear before other symptoms of

hyperglucagonemia. It is the presenting feature in 70% of patients with glucagonoma. In particular, glucagon-driven hepatic gluconeogenesis induces a later hypoacidemia, which is one of the causes favoring the onset of the skin lesions [123]. At the time of presentation, glucagonomas are commonly large, so that intraglandular localization is normally not problematic. In contrast to the even distribution of insulinomas throughout the pancreas, glucagonomas typically develop in the tail of the gland [111]. Concerning possible therapeutic options, the elective treatment of glucagonoma is the surgical resection of the lesion, possibly in combination with adjuvant chemotherapeutic protocols. In this respect, glucagonomas are typically associated with a good prognosis, even in the presence of liver metastases [124].

In addition to glucagonomas, also rare somatostatinomas are associated with diabetes and a hyperglycemic phenotype [125–127]. Somatostatin is secreted by a range of tissues, including the pancreatic islet delta cells, and particularly inhibits alpha and beta cell glucagon and insulin secretion in a paracrine manner, respectively. Also, somatostatin interferes with cholecystkinin-mediated release of pancreatic enzymes. Hypersecretion of somatostatin consequently presents with diabetes, malabsorption, steatorrhoea, and cholelithiasis due to reduced gallbladder contractility [128]. As these symptoms are relatively nonspecific, the majority of somatostatinomas are diagnosed incidentally and confirmed with a fasting somatostatin level $>$ 14 mol/L [128]. At the time of diagnosis, most cases of somatostatinomas are correlated with metastases [111].

The Reverse Connection: Type 2 Diabetes as a Risk Factor for Pancreatic Ductal Adenocarcinoma

Whereas endocrine neoplasms of the pancreas directly trigger imbalances in systemic glucose homeostasis and eventually lead to diabetes, epidemiological studies over the past decade have also established obesity-related type 2 diabetes as an important risk factor for exocrine pancreatic tumors, in particular pancreatic ductal adenocarcinoma (PDAC) [129]. Elevated levels of insulin represent a common feature of metabolic conditions associated with PDAC, such as obesity and type 2 diabetes mellitus. Indeed, insulin has been shown to directly stimulate pancreatic cancer cell growth, partly via the MAP kinase pathway, and to promote energy turnover in pancreatic cancer cells by inducing expression of specific glucose transporters [130]. These effects are further enhanced by high intrapancreatic insulin levels and by the expression of insulin as well as IGF-1 receptors on pancreatic cancer cells, thereby providing a distinct growth advantage to these cells [131]. Of note, even the cancer-promoting consequences of a high-fat diet might, at least in part, rely on compensatory beta cell proliferation and hyperinsulinemia in response to systemic insulin resistance [132].

In this regard, the general importance of beta cells for PDAC manifestation has been demonstrated by previous reports showing that specific destruction of this cell population by streptozotocin treatment protects hamsters from experimental pancreatic

cancer development [133]. Apart from the loss of growth-promoting insulin action, the beneficial effects of beta cell depletion on cancer development might also point toward a role of the endocrine cell compartment as a cellular precursor pool for PDAC, although this remains speculative in the setting of human PDAC [129].

Cancer cells take up high amounts of glucose, which is utilized for ATP production by aerobic glycolysis and generation of building blocks for nucleotide, amino acid, and lipid biosynthesis. Thus, increased concentrations of glucose in the circulation (hyperglycemia), as a hallmark of type 1 and type 2 diabetes mellitus, could contribute to tumorigenesis. Indeed, a number of epidemiological studies suggest that diabetes is associated with higher prevalence as well as increased mortality for certain types of cancer, including PDAC [134, 135]. The risk connection between diabetes and cancer is complex and might be based on various mechanisms including increased levels of proinflammatory cytokines as well as oncogenic effects of hyperglycemia which are not directly linked to glucose as an energy substrate, e.g., antiapoptosis, induced cell migration and invasion as well as hyperglycemic memory effects [136]. Despite the clear epidemiological connection between insulin resistance, obesity, and type 2 diabetes and PDAC development, neither the molecular mechanisms of insulin-dependent cancer growth nor the potential role of endocrine cells as the potential origin of ductal adenocarcinoma is fully understood to date, still providing a major challenge for biomedical research in the future.

Conclusion

Paraneoplastic syndromes are defined as signs and symptoms which present distant to the site of primary cancer or metastases. The best-understood syndromes result from tumor production of biologically active substances or, to a lesser extent, from autoimmune phenomena. The clinical symptoms vary widely and include systemic (fever, cachexia, etc.) and organ-specific manifestations (cutaneous, neurological, hematological, endocrine, etc.). In some cases, these can become the major clinical problems determining survival.

After successful treatment of the underlying malignant disease, paraneoplastic symptoms may resolve completely. Thus, early recognition of paraneoplastic syndromes is very important for the diagnosis of patients with pancreatic cancer, as is the follow up for monitoring disease progression.

Cross-References

- ▶ Apoptosis: Signaling Pathways in Pancreatic Cancer Pathogenesis
- ▶ Diagnostic Biomarkers
- ▶ EGFR (ErbB) Signaling Pathways in Pancreatic Cancer Pathogenesis
- ▶ Molecular Pathology of Carcinomas of the Ampullary/periampullary Region

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Diagnostic Biomarkers

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Contents

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Abstract

Pancreatic adenocarcinoma is the fourth leading cause of cancer death and the most deadly of all solid malignancies. Current methods for the early detection and diagnosis of pancreatic adenocarcinoma are largely ineffective and not feasible for uncovering small, often treatable precursor lesions in the general population. The discovery of biomarkers that aid in the early detection of pancreatic cancer would help to improve outcomes in patients and be of invaluable clinical benefit. This review discusses important considerations for the development of diagnostic biomarkers and profiles the promising molecular markers that have been evaluated in recent years.

Keywords

Pancreatic cancer · PanIN (pancreatic intraepithelial neoplasia) · IPMN (intraductal papillary mucinous neoplasm) · MCNs (mucinous cystic neoplasms) · CA19-9 · Circulating tumor DNA · EUS (endoscopic ultrasound) · Early detection · KRAS · Mutation · Pancreatic juice · Pancreatic cyst

Introduction

Pancreatic adenocarcinoma is the third leading cause of cancer death in the USA and is the most lethal of all solid malignancies. It is estimated that over 53,000 individuals will be diagnosed with pancreatic cancer in the USA in 2016, and nearly 42,000 will die from the disease [1]. The incidence of the disease has been increasing, and in the next few years, it is expected to be the second most common cause of cancer death in the USA [2]. Patients with pancreatic cancer often present late and respond poorly to chemotherapy and radiation, and the 5-year survival rate for this disease is currently less than 7%. Although imaging tests such as CT, EUS, and MRI can readily identify most pancreatic cancers, they can miss small cancers, and since even small pancreatic cancers of ~2 cm diameter are usually not curable, there is a need to be able to detect very small (subcentimeter pancreatic cancers) that have a greater

chance of being cured. Circulating tumor marker tests are used in clinical settings to help identify which patients would benefit from an imaging test.

Much effort has gone into identifying better diagnostic markers that could improve the detection of early-stage pancreatic cancer and its precursors. A suitable diagnostic marker test could be used to screen individuals at significantly increased risk of developing pancreatic cancer such as those with a strong family history of pancreatic cancer and those who carry germline mutations in pancreatic cancer susceptibility genes [3, 4]. Another high-risk group that is of interest to screen are older adults with new-onset diabetes [5].

Tumor markers have been defined as "a naturally occurring molecule that is measured in serum, plasma, other body fluids or in tissue extracts or paraffin-embedded tissue to identify the presence of cancer, to assess patient prognosis, or to monitor a patient's response to therapy with the overall goal of improving the clinical management of the patient" [6]. They may also be defined to include biological measurements such as the RECIST (Response Evaluation Criteria in Solid Tumors) imaging criteria for evaluating changes in tumor size with treatment. Thanks to improvement in data processing, the resolution of CT continues to improve, but CT is not currently being used as a screening test to detect pancreatic neoplasms primarily because of concerns about cumulative doses of radiation.

Pancreatic cancer tumor markers can be isolated from the blood, urine duodenal fluid, stool, and pancreatic tissue. Blood is advantageous given its ease of access and acceptability to the patient, but circulating marker levels are much lower in blood than in samples collected from the pancreas, often necessitating much more sensitive tests [7]. Pancreatic juice can be collected from the duodenum during an upper endoscopy with secretin stimulation. Pancreatic juice collection allows for biomarker analysis in patients with pancreatic abnormalities visualized by imaging but no defined mass or as a way to look at the pancreatic ductal system as part of screening protocols to detect microscopic neoplasia in individuals with an elevated risk of developing pancreatic cancer.

Pancreatic sampling is an invasive procedure, rendering it inappropriate as a screening tool for the general population. However, in patients at high-risk for developing pancreatic cancer, pancreatic juice sampling allows for sampling of abnormal areas with minimal side effects. This approach has been carried out in the Cancer of the Pancreas Screening (CAPS) studies [8, 9], in which patients with at least one first-degree and one second-degree relative with pancreatic cancer undergo pancreatic screening with EUS and MRI [10], generally beginning at age 55,to facilitate the early detection of asymptomatic pancreatic precursor lesions. There is suggestive evidence that pancreatic screening of high-risk individuals can improve outcomes [3, 4] (such as the detection of mostly resectable pancreatic cancers rather than advanced-stage cancers in screened patients and the detection of PanIN-3 and high-grade dysplasia in IPMN), but further studies are needed to evaluate long-term outcomes.

Combining molecular markers of pancreatic neoplasia with sensitive pancreatic imaging may ultimately prove to be a more effective screening tool for the early

detection of pancreatic cancer. Pancreatic cancer is the most deadly of all solid malignancies. Current therapies are largely ineffective once the disease has spread, emphasizing the need for accurate diagnostic biomarkers for asymptomatic precursor lesions. A variety of genetic, epigenetic, and protein changes occur as pancreatic neoplasms progress. Mutations, DNA methylation alterations, microRNAs, and protein alterations sampled from the blood, pancreatic juice, and cyst fluid have all been evaluated as potential diagnostic biomarkers for pancreatic cancer.

Using Diagnostic Tests to Identify Early-Stage Pancreatic Cancer

Pancreatic cancer is an almost universally lethal disease, but patients can be cured if precursor lesions are detected early and resected. Thus, there is considerable interest in designing a screening tool. Unfortunately, the majority of patients (>85%) are diagnosed with advanced, inoperable disease when current therapies are largely ineffective. Detecting pancreatic cancer in its earliest stages offers patients the best chance of being cured; however, there are a number of inherent challenges in identifying markers of pancreatic cancer precursors. Current imaging tests fail to detect small lesions that may progress to pancreatic cancer. Additionally, the prevalence of pancreatic precursor lesions increases with age, but many will never develop into pancreatic cancer. A comprehensive understanding of the genetic and histological differences that drive the formation and development of these different precursors is imperative as there are potentially significant consequences for both failing to identify precursor neoplasms and to over treating them. Furthermore, identifying diagnostic biomarkers to screen the general public for pancreatic cancer is largely impractical as the overall prevalence of this disease is low in the general population. While targeted screening of high-risk patients improves the positive predictive value of a screening test, this approach does not help improve the early detection of sporadic forms of pancreatic cancer. These and other important considerations for diagnostic biomarker development are discussed more comprehensively below.

Characterization of Pancreatic Cancer Precursor Lesions

A number of precursor lesions can give rise to pancreatic cancer. The most common of these neoplastic precursors, pancreatic intraepithelial neoplasm or PanIN, are microscopic lesions not readily detected by clinical imaging tests. The acquired genetic alterations that have been identified in pancreatic tumors have also been observed in PanINs, albeit at a lower prevalence [11]. The prevalence of PanIN-1 lesions increases with age in individuals without pancreatic disease; however, high-grade PanINs are typically observed in patients with invasive pancreatic cancer [12, 13]. In those patients with a family history of pancreatic cancer, resecting advanced PanIN lesions may be able to prevent the development of pancreatic cancer [14].

A second precursor neoplasm is the intraductal papillary mucinous neoplasm (IPMN). IPMNs are large, cystic neoplasms (≥1 cm) with a broad spectrum of

malignant potential. As pancreatic imaging technologies become increasingly sensitive, these lesions are more frequently diagnosed and treated [15]. Additionally, IPMNs are discovered incidentally in patients undergoing abdominal imaging [16]. Main-duct IPMNs have a higher malignant potential compared to branch-duct IPMNs. Branch-duct IPMNs are more likely to progress to invasive cancer if the lesions grow to >3 cm in size and are symptomatic or if they are associated with dilatation of the main pancreatic duct [17]. IPMNs are classified in a number of ways. There are histological subtypes, including gastric, intestinal, pancreaticobiliary, and mixed. They are also graded as either low grade (benign) or high grade (carcinoma in situ) (there is an emerging consensus among experts that the intermediate grade of dysplasia should be removed) [18]. A tumor marker's behavior can be expected to vary with the histological subtype and grade of IPMN lesion. Furthermore, while there are similarities in the genetic alteration characteristic of IPMNs, PanINs, and pancreatic cancer, there are also key genetic differences. High-grade PanIN lesions sometimes exhibit loss of *DPC4/SMAD4*, whereas IPMNs rarely inactivate this gene [19, 20].

A third, less common precursor neoplasm in the pancreas is the mucinous cystic neoplasm (MCN). MCNs, which are found more often in women, are cystic lesions that produce mucin and are defined by their ovarian-type, fibrous stroma. The ability to differentiate cystic lesions with varying malignant potential is important, as patients who undergo a pancreatic resection for IPMNs or MCNs that do not have infiltrating pancreatic adenocarcinoma are usually cured. If left untreated, these lesions can progress to invasive carcinoma. Mucinous cysts that have malignant potential require surveillance, whereas some cysts have little or no malignant potential such as serous cystadenomas and pseudocysts. Distinguishing these cysts by imaging and cyst fluid markers is useful as it determines how these lesions should be followed.

Distinguishing Benign Lesions from Precursor Neoplasms

As suggested above, not all pancreatic lesions have the same propensity to develop into infiltrative pancreatic cancer. It is estimated that approximately 20% of pancreatic cysts that are removed are found to be benign [21, 22]. In light of the fact that surgical resections of pancreatic lesions are associated with significant morbidity, considerable effort has been put forth to design better methods of distinguishing truly benign and low-grade lesions from high-grade precursors that warrant treatment. Exome sequencing analysis of pancreatic lesions has begun to delineate patterns of genetic alteration characteristic to each cystic precursor type. For example, IPMNs and MCNs frequently exhibit mutations in *KRAS*, *RNF43*, *TP53*, and *CDKN2A*, IPMNs but not MCNs frequently harbor mutations in *GNAS* [23, 24], and both MCNs and IPMNs can harbor *SMAD4* mutations, but this is often a late event often only seen in the invasive component [19, 25]. In contrast, the more indolent serous cystadenomas harbor mutations in *VHL* and solid pseudopapillary neoplasms, a rare neoplasm usually found in young people harbors*CTNN1* [23, 24].

Differentiating PanIN lesions that will ultimately give rise to infiltrative adenocarcinoma from those that will not progress has proven to be a more challenging task. The prevalence of low-grade PanINs increases with increasing age in patients without pancreatic disease; however, Terhune and colleagues estimate that over 99% of these lesions will never develop into invasive cancer [26].To date, the only way to accurately characterize PanINs is to remove them for histological evaluation. As imaging technologies become more advanced, and the detection of smaller lesions becomes possible, an even greater need will exist for tools to distinguish harmless precursors from high-grade lesions. See also the chapter on the ▶ "The Molecular Pathology of Precursor Lesions of Pancreatic Cancer".

Identifying High-Risk Patients for Screening

While there is considerable excitement that advancements in imaging capabilities and molecular diagnostics should lead to better detection and classification of pancreatic precursors, it remains challenging to develop a screening tool that could be utilized broadly. Although the incidence of pancreatic cancer is increasing and the lifetime risk of developing the disease is ~1.5% in the USA, the incidence of the disease at any one point in time is much lower. Even if a screening test were to be developed with 95% sensitivity and specificity, a significant number of patients would be falsely identified as positive, and they would subsequently be subjected to further evaluation.

Targeting populations with a higher prevalence of pancreatic cancer (those with an increased risk for developing the disease) improves the positive predictive value of a diagnostic test. High-risk groups with a significantly higher risk of developing pancreatic cancer include those with a family history of the disease, particularly those multiple first-degree relatives who have been diagnosed with pancreatic cancer [14, 27]. Additionally, patients with germline mutations in genes such as *BRCA2, ATM*, *CDKN2A*, and *PALB2*, as well as individuals with inherited causes of recurrent acute pancreatitis such as from germline *PRSS1*mutations, are also at increased risk for developing pancreatic cancer [28, 29]. Ultimately, as the goal of screening is to identify and treat precursor lesions before they progress into invasive cancer, patients at increased risk for developing this disease are likely to benefit greatly from improvements in molecular diagnostics and imaging. Screening high-risk populations enables the opportunity to detect more stage I cancers. Studying biomarker behavior in the main setting where a screening blood test for high-risk individuals is the best way to evaluate the test. See also the chapter on ▶ "Familial Pancreatic Cancer" screening for inherited pancreatic cancer.

Implications of Disease Heterogeneity

Pancreatic cancer is a highly heterogeneous disease, with distinctive pathological, molecular, and clinical presentations. For example, pancreatic adenocarcinomas that

arise from IPMNs differ molecularly from those that progress from PanINs [19]. Certain variants, such as those with medullary histology, frequently exhibit microsatellite instability and lack mutations in a common driver of pancreatic cancer, *KRAS* [30]. Others have classified pancreatic cancers by their etiologies or pattern of inheritance; however, it is not yet understood whether such distinctions aid in predicting either tumor or tumor marker behavior. For example, hereditary gastric and colorectal cancers are pathologically distinct from sporadic tumors at these sites. In pancreatic cancer, preliminary data suggests that familial and sporadic pancreatic adenocarcinomas are very similar at the genetic and epigenetic level, but additional studies are necessary to further define the molecular and pathological profiles of familial and sporadic pancreatic cancer [31, 32]. Given the degree of molecular [33] and pathological heterogeneity inherent in pancreatic cancers, it is unlikely that a single tumor marker will be accurate and sensitive enough to distinguish each of the variants of this disease.

Importance of Disease Controls and Early-Stage Samples in Assessing Biomarker Behavior

In addition to pathological and genetic heterogeneity, the clinical presentation of pancreatic cancer can be variable, which may in turn influence tumor biomarker behavior. As pancreatic cancer progresses, patients may suffer from secondary complications from their disease such as cachexia, diabetes, and obstructive jaundice. Differences in the clinical manifestations in pancreatic cancer are likely a result of differences in the pathophysiology of the disease. Importantly, proteomic, epigenetic, and expression changes are likely to occur in response to these complications and could be identified as novel diagnostic biomarkers in preliminary studies. However, many of these candidate markers will lack the appropriate specificity.

For this reason, it is necessary to include the appropriate disease controls for diseases that can mimic or coexist with pancreatic cancer in studies evaluating novel diagnostic biomarkers for pancreatic cancer. For example, obstructive jaundice is observed frequently in patients with pancreatic cancer, but many studies fail to include individuals that develop obstructive jaundice in the absence of pancreatic cancer. CA19-9, the current gold standard for pancreatic diagnostic markers, is known to be elevated in patients with benign causes of obstructive jaundice.

Another important disease control group for pancreatic cancer marker studies are patients with diabetes, as nearly 25% of patients have this condition when diagnosed with pancreatic cancer. An additional 40% will have impaired glucose tolerance [34, 35]. It remains uncertain as to whether the pancreatic cancer causes the development of diabetes in these patients, although surgical resection of the tumor often results in curing the patient of this metabolic condition. There is growing interest in screening older patients with new-onset diabetes, as it may lead to the early detection of asymptomatic and early-stage pancreatic cancer in these patients [36].Metabolic syndrome, a very common condition in the population and an important risk factor for pancreatic cancer [37], is likely to exert an important influence on many candidate biomarkers.

Chronic pancreatitis is another condition that can mimic pancreatic cancer. Areas of focal pancreatitis can resemble a pancreatic cancer with pancreatic imaging tests used in clinical practice, and patients may have to undergo surgical resection to receive an accurate diagnosis. Individuals with autoimmune pancreatitis often exhibit symptoms that are similar to those observed in patients with pancreatic cancer, such as an enlarged pancreas with common bile duct obstruction [38]. In addition to mimicking the clinical manifestations of pancreatic cancer, chronic pancreatitis is a known risk factor for the development of pancreatic cancer. This increased risk is particularly noteworthy in patients who develop pancreatitis at a young age or those with an inherited form of the disease [39]. Many potential markers can accurately distinguish patients with pancreatic cancer from healthy controls, but they cannot reliably differentiate individuals with chronic pancreatitis. For example, CA19-9 can be elevated in patients with chronic pancreatitis. This may not necessarily mean the biomarker would not have diagnostic value, because the clinical syndrome of chronic pancreatitis is not common in the population. However, many more patients have chronic inflammation from other causes, and the inability of a biomarker to distinguish chronic pancreatitis from pancreatic cancer raises the likelihood that the biomarker will not be as specific in patients with other inflammatory comorbidities. In some diagnostic scenarios, it is important to be to distinguish between these two conditions. For example, pancreatic imaging may identify nonspecific abnormalities that could be due to focal areas of pancreatitis or to a neoplasm. A diagnostic biomarker applied to pancreatic samples should be able to distinguish these two pathologies but often cannot. For example, low concentrations of mutant *KRAS* are found in pancreatic juice samples from patients undergoing screening for their family history of pancreatic cancer, from patients with chronic pancreatitis, and even occasionally in patients without known pancreatic disease [40]. This mutant *KRAS* is thought to arise from microscopic PanIN lesions.

An important challenge to evaluating diagnostic biomarkers is that there are few patients who are enrolled in screening studies in the diagnostic setting where an early detection test would be applied. Instead, biomarker studies evaluate the candidate biomarker performance in patients with pancreatic cancer, and most of these patients have advanced-stage disease. As pancreatic cancer spreads, secondary changes including inflammation, fibrosis, weight loss, obstructive jaundice, and diabetes arise that affect biomarkers. Many candidate markers identified in late-stage disease turn out to reflect these secondary metabolic changes. Such biomarkers will not have good diagnostic utility in the screening setting.

Evaluating Diagnostic Biomarker Candidates

Several sets of guidelines are available to help clinicians and investigators assess diagnostic biomarkers. For example, the Quality Assessment of Diagnostic Accuracy Studies (QUADAS) and the Standards for Reporting of Diagnostic Accuracy (STARD) are guidelines for evaluating diagnostic markers and reporting diagnostic accuracy [41]. When assessing candidate diagnostic biomarkers, it is imperative to

consider both the benefits and potential problems with implementing a potential marker in clinical practice. The utility of any diagnostic marker depends on the clinical setting it is employed in. Thus, the performance requirements for a diagnostic marker will vary depending on factors such as the accessibility of the biological sample, the clinical question being addressed, and the implications of the results of the test. For pancreatic cancer, tumor markers can be assayed from blood, stool, pancreatic tissues, and fluids such as cyst fluid and pancreatic juice. Blood is advantageous given its ease of access and acceptability to the patient, but circulating marker levels are much lower in blood than in samples collected from the pancreas, often necessitating much more sensitive tests [7]. Additionally, levels of some tumor markers may not become detectable in the blood until invasive carcinoma has developed. An example of this point is CA19-9, which is elevated in >80% of patients with late-stage disease. In contrast, only ~65% of patients with early-stage pancreatic cancer have increased levels of CA19-9 [42].

When assessing the clinical utility of a diagnostic screening tool, it is important to understand the settings in which it will be applied. For example, when a patient presents with symptoms that are strongly suggestive of pancreatic cancer, pancreatic imaging is done. A pancreatic protocol CT scan will often identify a pancreatic neoplasm, and further marker studies would not be necessary. In contrast, a patient with nonspecific symptoms would benefit from a highly accurate blood test, as more invasive testing when the probability of a cancer diagnosis is low would not be justified. In light of the criteria and considerations outlined above, the remainder of this chapter will be devoted to examining the most important candidate biomarkers that have been assessed to aid in the diagnosis of pancreatic cancer.

Blood-Based Biomarkers for Early Detection

A diagnostic biomarker for pancreatic cancer can be measured in many biological fluids including blood and urine, pancreatic cyst fluid, pancreatic juice, pancreatic tissue, and stool. While developing a circulating biomarker would have a lot of clinical utility, circulating tumor markers are generally found at significantly lower concentrations in the blood compared to other biological samples, necessitating an assay that is highly accurate at detecting very low levels of analyte [7]. If the goal is to screen asymptomatic patients with microscopic precursor lesions, this is likely not even possible with a blood test as tiny precursor lesions likely do not shed detectable levels of candidate biomarkers into the bloodstream. Indeed, it has been estimated that several billion neoplastic cells (a pure tumor mass of ~2 mm diameter) are needed to elevate levels of a typical circulating biomarker [43]. Since much of a pancreatic cancer cell mass consists of non-neoplastic stroma, for pancreatic cancer this estimate would correspond to pancreatic tumor mass of at least 3–4 mm. Despite these challenges, considerable effort has been expended to identify circulating biomarkers that could improve the early detection of pancreatic cancer in patients.

CA 19-9

Despite its limitations, carbohydrate antigen 19-9 (CA19–9) remains the gold standard for pancreatic adenocarcinoma tumor markers. CA19-9 is a sialylated lacto-N-fucopentaose II related to the Lewis[a] blood group antigen on MUC-1 and is recognized by a specific monoclonal antibody [44]. The CA19-9 antigen is relatively specific for pancreatic cancer but is seen in benign conditions such as chronic and acute pancreatitis, biliary obstruction, cirrhosis, cholangitis, and cholecystitis. Levels can also be elevated in non-pancreatic malignancies like ovarian and colorectal cancer [45, 46]. Furthermore, up to 10% of individuals do not express the Lewis blood group antigen and thus would not have measureable CA 19-9 levels [6]. Importantly, although CA 19-9 levels are elevated in ~80% of patients with advanced pancreatic cancer, it is elevated in only ~60% of patients with resectable disease, and most of these patients already have lymph node metastases [46]. Indeed, high CA19-9 levels at diagnosis predict poor outcome [46]. For these reasons, CA 19-9 has largely been used to monitor patient's responses to therapy rather than as a diagnostic marker [46]. Multiple societies have all issued guidelines for its use in patients with pancreatic cancer [47, 48]. Despite its limitations, several studies have evaluated the utility of using CA19-9 as a test for the early detection of pancreatic cancer. Patient cohorts have been used to examine CA19-9 behavior before clinical diagnosis. Many patients will have elevated CA19-9 1 year or more prior to a clinical diagnosis of pancreatic cancer, but it is suspected that many of these patients already have advanced disease [49, 50].

In an attempt to improve the diagnostic utility of CA19-9, some investigators have determined if it would be better to have a test that targeted other modified carbohydrate antigens on MUC-1 or other proteins, but to date none of these biomarkers have been found to be more effective than CA19-9.

Combining CA 19-9 with Other Markers

Many groups have explored whether combining other markers with CA19-9 can improve the sensitivity and specificity of a screening test for patients. Typically these studies initially evaluate new markers in the setting of advanced pancreatic cancer since it is more difficult to recruit sufficient patients with early-stage disease.

Some of the better performing markers have been evaluated in combination with CA19-9. These markers include serum CEA, MIC-1, TIMP-1, HIP (PAP or REG3A), and others [51, 52] and were often identified as candidate biomarkers by comparing pancreatic tumor vs. normal pancreas samples for alterations in gene expression. One such marker is MIC-1 (macrophage inhibitory cytokine 1) a member of the TGF-ß super family of proteins, which is overexpressed in primary pancreatic cancers and is elevated in the serum of patients with resectable pancreatic cancer. The combination of serumMIC-1 and CA19-9 achieved higher diagnostic accuracy over using either marker alone (AUC 0.87) [42].

Brand examined a panel of markers and found that combining CA19-9 with carcinoembryonic antigen (CEA) and TIMP-1 could differentiate patients with

mostly advanced pancreatic cancer from those with benign disease with a sensitivity/specificity of 76% and 90%, respectively [52].

Some investigators are exploring the value of using large panels of protein markers as a diagnostic test. A multi-marker circulating panel has been shown to significantly improve the diagnosis of pancreatic cancer compared to CA19-9 alone [53], but it remains to be seen if such approaches can improve the diagnosis of very-early-stage pancreatic cancer.

Since many circulating proteins are shed in the urine, this sample has been evaluated as a source of biomarkers. In one study, Costello et al. used a mass spectrometry approach to identify novel biomarkers and identified a thre-protein panel that had good ability to distinguish patients with pancreatic cancer from controls [54]. Further studies are needed to evaluate if a urine biomarker panel could have diagnostic utility. (see also the chapter on the "▶ Development of Novel Diagnostic Pancreatic Tumor Biomarkers").

DNA Mutations

Detecting somatic mutations has the advantage that these mutations are not normal, so in principle, the presence of these somatic mutations should reflect disease. In contrast, most other markers have a normal range, and their value as diagnostic markers is closely related to their concentration. However, somatic mutations generally emerge in benign neoplasms, so somatic mutations detected in pancreatic samples often reflect the presence of benign neoplasms. In the blood, the presence of somatically mutated DNA is very concerning for the presence of cancer. DNA shed from cancer cells can be detected in the blood as cell-free circulating tumor DNA (ctDNA). Some cancer types shed relatively large amounts of ctDNA into the circulation (~1% of total DNA), particularly with advanced disease, others such as pancreatic cancer typically shed very low amounts of ctDNA (<0.1%) [55]. Until recently, the difficulty of detecting low concentrations of mutant DNA in the circulation has limited their utility as diagnostic biomarkers. Over 90% of invasive pancreatic adenocarcinomas harbor mutations in *KRAS*. ctDNA tests rely on PCR amplification to detect mutant DNA, and these tests can generate false-positive results that approach the levels seen in the circulation [56]. For this reason, it is particularly important that studies involving ctDNA employ extensive testing to determine the specificity of their assay.

Since levels of circulating tumor DNA are low, highly sensitive and specific technologies are needed to detect these mutations. Several useful strategies have been developed to detect ctDNA. Kinde and colleagues employed an approach termed Safe-Sequencing System (SafeSeqS). It involves assigning a unique identification DNA sequence (UID) to each fragment of DNA followed by an amplification step that produces UID families. When greater than 95% of a UID family contains the same mutation, it is termed a super mutant. Thus, a true mutation would be present in nearly all DNA fragments with the UID and give rise to a super mutant, whereas a mutation that occurs during amplification would not

[56]. Utilizing SafeSeqS, mutant *KRAS* was detected in the plasma of 85% of patients with advanced pancreatic cancer but only 45% of individuals with localized disease [55].Digital droplet PCR (ddPCR) technology has also been used to detect mutant *KRAS* ctDNA [57, 58] in patients with pancreatic cancer and has the advantage of being a simpler method but has the limitation that a specific probe is required for each mutation of interest. One small study found that ctDNA can be detected in patients with IPMNs, but this study included only small numbers of control patients and needs to be confirmed [59].

The challenges of reliably detecting low levels of ctDNA has limited its evaluation as a potential screening test for pancreatic and other cancers to date, but it is likely to become a useful test in the future. Since most patients with early-stage pancreatic cancer have not been found to have detectable ctDNA with existing technologies, ctDNA cannot be relied upon as a diagnostic test but could be a useful adjunct. ctDNA detection is being evaluated as a tumor marker to monitor early recurrence and tumor burden [58, 60], and it is expected to become a useful clinical test in this setting.

Circulating Tumor Cells (CTCs)

CTCs are intact cells that contain nucleic acids and can be separated from normal cells within the circulation [61]. Although the term CTCs implies that the cells being detected are cancer cells, many use the term CTC for all circulating cells expressing epithelial markers despite the fact that these cells are also found in many patients who do not have cancer [62, 63]. For this reason, tests are being developed to selectively isolate cancer cells and not all circulating epithelial cells. Such tests rely on either flow cytometry separation of cells based on surface molecules, selection based on size using microfluidic chips or filter-based methods, or molecular characterization of isolated cells. In patients with pancreatic cancer, CTCs are usually detected in patients with advanced-stage disease rather than early-stage disease suggesting that CTC-based tests are likely to have more value in disease monitoring rather than patient diagnosis. See also the chapter on ▶ "Circulating Tumor Cells".

Other circulating biomarkers have been evaluated as possible diagnostic markers including microRNAs and other noncoding RNAs, exosomes, and autoantibodies.

RNA Biomarkers

In addition to interrogating tumor DNA as potential diagnostic biomarkers for pancreatic cancer, many groups have begun to look closer at circulating RNA molecules. While most types of RNA molecules are subject to rapid degradation by RNases, microRNAs (miRNAs) are more stable. In addition, RNA molecules can be protected from RNases if they are incorporated into extracellular vesicles.

microRNAs

MicroRNAs (miRNAs) are small, noncoding RNAs that regulate gene expression. Derived from larger RNA transcripts that are degraded by the enzyme DICER,

miRNAs then associate with the RNA-induced silencing complex (RISC) and bind to the 3′ untranslated regions of a gene. This results in either RNA degradation or translational repression. In cancer, miRNA expression tends to be decreased; however, there are several miRNAs that have been identified as overexpressed and could therefore be potentially targeted as diagnostic markers. Numerous miRNAs including miR-21, miR-155, miR-196, miR-200, miR-1246, and miR-1290 have been identified as overexpressed in pancreatic adenocarcinomas. Several studies have measured circulating miRNA levels to determine its potential as a diagnostic test. Li et al. identified miR-1290 as having good diagnostic performance [64], and similar results were found for the noncoding RNA RNU2 which has close homology to miR-1246 and miR-1290 [65]. Several of these miRNAs are elevated in other cancers, so while it is possible that certain miRNAs could have value for a pan-cancer screening test, they are not likely to serve as a diagnostic test for a specific cancer type. Despite these initial studies, there has been a lack of uniformity in the results of different studies that is thought to reflect a variety of challenges related to assay performance and study design that need to be overcome before miRNA can be used as diagnostic tests.

Exosomes

There is growing interest in the potential uses of extracellular vesicles such as exosomes as diagnostic, prognostic, and therapeutic biomarkers for a variety of diseases. Exosomes are small, membrane vesicles between 30–100 nm in size that are secreted by many cell types and commonly express the tetraspanin molecules CD9, CD63, and CD81. Exosomes are metabolically active and express surface molecules that allow them to communicate with and influence the behavior of distant cells and tissues. They are known to carry various macromolecules including proteins and nucleic acids and so could be a valuable source of cancer biomarkers. Most circulating exosomes are thought to derive from platelets so there is considerable interest in identifying cancer-specific exosomes. Melo et al. recently reported that it may be possible to differentiate pancreatic cancer-derived exosomes based on increased expression of glypican-1 (GPC1), but problems with this study necessitate that further studies are needed before this biomarker can be considered promising [66]. It remains to be determined whether there is a test that could specifically detect exosomes released from pancreatic cancer or pancreatic precursor lesions into the circulation that could be used as a diagnostic test (see also chapter on ► "Cancer Exosomes for Early Pancreatic Cancer Diagnosis and Role in Metastasis").

Autoantibodies

Autoantibodies are generated from the recognition of the antigens arising from mutant gene products as well as novel splice variants and proteins. Serum autoantibodies have been observed in patients with a variety of different tumor types and are sometimes observed prior to a cancer diagnosis. Autoantibodies to p53 protein are among the most common autoantibodies detected in patients with cancer, but overall

autoantibodies are only detected in a minority of patients with cancer. It remains to be seen whether a panel of autoantibodies can be used as an aid to diagnosis.

Pancreatic Cyst Fluid Markers

Pancreatic cysts are commonly identified as incidental findings among patients undergoing abdominal imaging. The prevalence of these increases with age with over 10% of individuals over age 70 having one or more pancreatic cysts [67]. Most of these pancreatic cysts are thought to be IPMNs. Since some pancreatic cancers arise from cysts, it is important to determine the neoplastic nature of a pancreatic cyst, because it can provide an opportunity to intervene to treat these lesions before they are fatal. However, only a small minority of pancreatic cysts become malignant, and effective diagnostic tests are needed to classify and grade pancreatic cysts for their malignant potential. The imaging characteristics of a cyst are helpful in determining cyst pathology and the likely presence of malignancy. Guidelines have been developed based on expert opinion, such as the Fukuoka guidelines, that rely on pancreatic imaging to help clinicians determine who requires surveillance and who requires an operation [68]. However, these guidelines are not sufficient enough to predict the neoplastic nature of a cyst [69, 70]. Biomarkers that could better classify and grade the neoplastic nature of a pancreatic cyst are needed to minimize over-or undertreatment. In general IPMNs with low-grade dysplasia can be monitored, but IPMNs with high-grade dysplasia or an associated invasive cancer require treatment. It is not yet clear that a circulating biomarker could be used as a test that indicates the presence of an IPMN. In principle, circulating blood tests could in principle identify the emergence of invasive cancer in patients with cysts, but cyst fluid sampling using EUS-guided fine needle aspiration (EUS-FNA) is more likely to be informative. Cyst fluid can be evaluated for the presence of protein markers such as CEA, but newer markers such as mutated and methylated DNA, miRNAs, mucins, telomerase activity, and other markers have been examined in cyst fluid for their potential as diagnostic biomarkers.

Cyst Fluid DNA Markers

Since mucinous neoplasms harbor mutated DNA, the diagnostic utility of cyst fluid mutant DNA marker panels has been evaluated in multiple studies.

Exome sequencing analysis of pancreatic cystic neoplasms has been very helpful to identify the genes mutated in each type of neoplastic cyst, and this information has been very useful for cyst fluid biomarker studies. *GNAS* mutations are commonly found in IPMNs (~60–70% of lesions) and are very specific for IPMN compared to other types of pancreatic cysts [24]. In addition to mutations in *GNAS* and *KRAS* (one of these mutations are found in over 90% of IPMNs), IPMNs frequently exhibit mutations in *RNF43*, *TP53*, and *CDKN2A*. In contrast, the more indolent serous

cystadenomas and solid pseudo papillary neoplasms harbor mutations in *VHL* and *CTNN1*, respectively [23, 24]. In keeping with these results, studies have demonstrated that the detection of a *KRAS or GNAS* mutation in cyst fluid is specific marker that indicates the presence of a mucinous cyst, but because these mutations generally arise relatively early in the evolution of a cystic neoplasm, the detection of these mutations in cyst fluid does not reliably distinguish benign and malignant pancreatic cysts [71].

Molecular analysis of cyst fluid samples using next-generation sequencing assays designed to detect low-abundance mutations across a panel of genes as well as chromosomal alterations can accurately distinguish mucinous from non-mucinous cysts and has a very good ability to gauge the neoplastic grade of a pancreatic cyst [72].

Telomerase Activity

Telomere length dictates how long a cell will live, as short telomeres act as signals to the cell to stop dividing and enter senescence. The enzyme telomerase adds telomere repeat sequences to the ends of telomeres. Without it, telomeres would eventually shorten to critical levels and result in the fusion of chromosome ends. Telomerase is typically inactive in somatic cells but becomes active mainly in stem cells, as well as most cancer cells and precancerous cells with high-grade dysplasia.

Hata and colleagues recently evaluated the diagnostic performance of cyst fluid telomerase activity measured using an assay that relies on digital droplet technology and the telomerase repeat amplification protocol (TRAP) assay. Among cyst fluid samples from the surgical resections from 219 patients, telomerase activity had an overall diagnostic accuracy of 88% for distinguishing cysts with high-grade dysplasia/invasive cancer from those with lower grades of dysplasia and those without dysplasia. The diagnostic performance of telomerase activity for cysts that were characterized as having "worrisome features" was also high (AUC of 0.84) [73].

Aberrant DNA Methylation

A variety of genome-wide methylation studies have been done to identify aberrantly methylated genes in pancreatic cancers and precursor lesions. Initial studies evaluating the diagnostic performance of a panel of DNA methylation markers in cyst fluid have found that they have promising diagnostic utility.

Other Cyst Fluid Markers

Mucinous neoplasms produce abundant mucin, and in preliminary studies mucin profiles of cystic neoplasms have been found to discriminate between mucinous cysts from non-mucinous cysts [74]. MicroRNA alterations arise in IPMNs and other cystic neoplasms, but further studies are needed to better evaluate their

diagnostic utility. One protein biomarker of IPMNs known as mDas has been shown to be overexpressed primarily in IPMNs of intermediate and high-grade dysplasia, and in preliminary studies cyst fluid mDas levels predicted the grade of dysplasia of an IPMN with very good accuracy [75]. See also the chapter on the "► Management of Cystic Neoplasms of the Pancreas Including IPMNs."

Diagnostic Markers for Pancreatic Juice

Pancreatic juice tests are being evaluated as an adjunct to endoscopic evaluation of the pancreas. Pancreatic juice is collected after secretin infusion when patients are undergoing an endoscopic ultrasound as part of their pancreatic screening evaluation. In the pancreatic screening setting, pancreatic juice is collected from the duodenal lumen. Purer pancreatic juice samples can be obtained during an ERCP procedure, but this test is too invasive for routine use in the screening setting.

Since mutation concentrations in pancreatic juice collected from the duodenum are very low (0.1–1%), sensitive mutation detection technologies have been employed to detect mutations. Using secretin-stimulated pancreatic juice samples from patients enrolled in the CAPS study, Kanda and colleagues assayed *GNAS* mutations and found that their detection was highly specific for the presence of a pancreatic cyst, highlighting the utility of duodenal collections of pancreatic juice as a source of markers of pancreatic ductal neoplasia [76]. Subsequent studies found that *p53* mutations in duodenal collections of pancreatic juice were found only in patients with high-grade lesions and invasive ductal adenocarcinoma [77]. Among patients undergoing pancreatic screening, *KRAS* mutations are commonly detected, even in patients without pancreatic cysts; these mutations are also occasionally detected in patients without any suspicion of pancreatic disease and are thought to reflect the presence of mostly low-grade PanIN in these patients. However, mutant *KRAS* DNA was also detected in 19% of control patients [40]. More recently, next-generation sequencing technology has been employed to detect a panel of mutations in pancreatic juice. Since mutations are present at very low concentrations in pancreatic juice samples, a digital next-generation sequencing method was used which found that overall mutation concentrations and in particular *SMAD4* and *TP53* mutations were very useful at distinguishing patients with pancreatic cancer from those with IPMN and normal pancreata [78].

In addition to the genetic mutations that have been described above, epigenetic alterations (DNA methylation alterations) are common in pancreatic cancer. Initial studies evaluated these biomarkers in pure pancreatic juice samples isolated during ERCP. Subsequent studies have investigated candidate pancreatic cancer DNA methylation markers in pancreatic fluid samples collected from the duodenum. Further studies are needed to evaluate whether such a test could be used to evaluate the pancreas of patients undergoing pancreatic screening and surveillance.

Biomarkers as Molecular Imaging Targets

Molecular imaging approaches are being developed for imaging small cancers [79]. Several targets have been evaluated in preclinical models to determine if they could improve the molecular imaging of pancreatic cancer or its precursors. Candidate targets that have been evaluated are overexpressed membrane proteins, but more studies are needed.

Conclusions

Pancreatic cancer is an almost universally lethal disease, with most patients developing symptoms only after metastasis has occurred. Early detection and surgical resection offer the best chance for a cure, but this necessitates the development of a screening tool that can detect asymptomatic precursor lesions. While progress has been made in the characterization of genetic and epigenetic alterations in pancreatic precursor lesions and pancreatic cancer, the current gold standard for clinical diagnostic biomarkers is still CA19-9.As the prevalence of this disease is very low in the general population, it is particularly challenging to identify markers with a high enough specificity to avoid unacceptably high false-positive rates. Screening high-risk individuals has aided in identifying novel candidate diagnostic biomarkers. As not all potential markers are able to adequately distinguish pancreatic cancer from diseases that mimic it (chronic pancreatitis), carefully designed studies with the correct disease controls are essential. As imaging technologies become more sensitive and pancreatic lesions are increasingly found, it will be important for clinicians to be able to distinguish lesions with high malignant potential from those that will likely never progress to cancer. A number of promising markers have been identified in recent years, and further rigorous investigation into their diagnostic potential is necessary to improve the early detection of this disease.

Key Summary Points

- Pancreatic adenocarcinoma is the third leading cause of cancer death in the USA and is the most lethal of all solid malignancies.
- Tumor markers are naturally occurring molecules that can be used to identify cancer, assess a patient's prognosis, and monitor their response to therapy.
- There are currently no biomarkers recommended for general population screening. Individuals with a sufficiently increased risk for developing pancreatic cancer can undergo screening with EUS and MRI once they reach the appropriate age. Since it is not certain how beneficial pancreatic screening is, pancreatic screening is best undertaken as part of a research study.
- Despite its limitations as a diagnostic biomarker, CA19-9 is still the gold standard circulating pancreatic cancer biomarker against which other markers are evaluated.

- The accurate detection of circulating tumor DNA such as mutant KRAS DNA could be a useful test for the early diagnosis of pancreatic cancer but more studies are needed. Other biomarkers are still investigational. A current limitation of many studies evaluating candidate diagnostic biomarkers is that biomarker performance is not evaluated in the early detection setting, due the paucity of patients with stage I disease. Initial studies of candidate diagnostic biomarkers often do not enroll demographically matched enough disease controls to account for how biomarkers will perform in the clinical setting in which the biomarker would be used.
- It is likely that advancements in the early detection of pancreatic cancer will come as a result of screening high-risk patients with pancreatic imaging coupled with markers that sensitive and specific at detecting stage I pancreatic cancer and PanIN-3.

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Pancreatic Adenocarcinoma: CT and PET/CT

Götz M. Richter

Contents

Abstract

During the last years, startling epidemiologic facts find pancreatic adenocarcinoma to be on the rise with rapidly increasing relevance for public health. Recent projections for the year 2030 predict pancreatic adenocarcinoma to range among the top three deadly cancers in the Western world (Matrisian and Berlin, Am Soc Clin Oncol Educ Book 35:e205–215, 2016; Rahib et al., Cancer Res

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74:2913–2921, 2014). As a result, early detection, correct staging, and adequate peri- and posttherapeutic imaging strategies must play a very important role in present and future oncology in general and in pancreatic adenocarcinoma in particular. This will be outlined and discussed in this chapter. Early tumor detection is one of the key factors for a potential cure by surgical resection. Major advances in MDCT (multidetector computed tomography), including 2D and 3D reconstruction, are highly useful in improving staging and postsurgical care. PET-CT is particularly helpful in differentiating between malignant and benign in complex clinical problems such as discriminating between autoimmune pancreatitis and pancreatic adenocarcinoma, identifying distant metastastic disease in the pretheraupeutic staging workup, and, furthermore, discriminating between benign fibrotic tissue and tumor recurrence in the follow-up after surgical resection.

Keywords
MDCT (multidetector computed tomography) · PET (positron emission tomography) · MRI (magnetic resonance imaging) · Pancreatic adenocarcinoma · Stromal desmoplasia · Hypoattenuation · Resectability · Vascular invasion · Pancreatic duct occlusion · Indeterminate lesion · Autoimmune pancreatitis · Standardized reporting protocols

During the last years, startling epidemiologic facts find pancreatic adenocarcinoma to be on the rise with rapidly increasing relevance for public health. Recent projections for the year 2030 predict pancreatic adenocarcinoma to range among the top three deadly cancers in the Western world [1, 2]. As a result, early detection, correct staging, and adequate peri- and posttherapeutic imaging strategies must play a very important role in present and future oncology. This will be outlined and discussed in the following chapters.

Primary Imaging and Tumor Detection

Starting at the early nineties with the advent of multidetector computed tomography (MDCT), primary diagnosis of pancreatic adenocarcinoma has become feasible at tumor sizes below 3 cm and, furthermore, including a better depiction of involved organs and adjacent structures [3–5] (Figs. 1, 2, 3, 4, and 5). This came parallel to advances in surgical strategies to improve the dismal prognosis of advanced tumor stages of pancreatic adenocarcinoma. Already in 1996, Conlon described the long-term survival of up to 20% in patients with potentially resectable stages. Hence, adequate progress in imaging and, specifically, early detection is crucial for improving life expectancy [6]. During the last decade and with the widespread availability of either dual-source CT imaging or at least advanced multidetector systems ($\geq$ 256), modern MDCT provides improved accuracy in early tumor detection which in

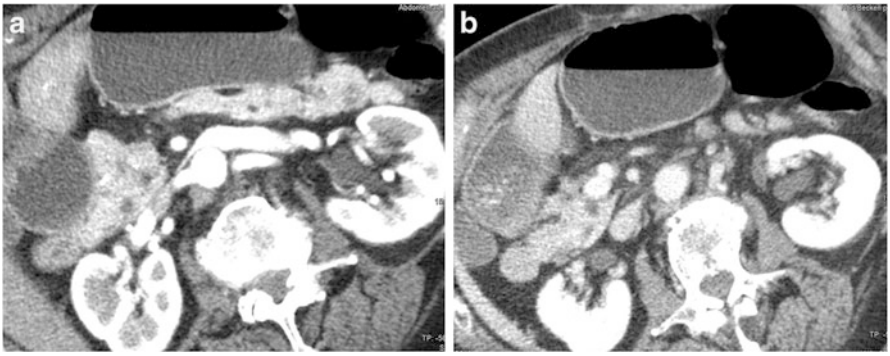

Fig. 1 T1 pancreatic adenocarcinoma of the head. The hypoattenuation sign. (**a**) arterial phase MDCT. Small hypoattenuating focal lesion immediately dorsal to the superior mesenteric vein without infiltration signs. Note the distinct contrast uptake between normal surrounding pancreatic tissue during arterial phase: high in normal low in tumor parenchyma. (**b**) Venous phase MDCT. Slightly better lesions conspicuity compared to the arterial phase. The small hypoattenuating focal lesion immediately dorsal to the superior mesenteric vein is relative sharply demarcated (not a regular finding). Note again the distinct contrast uptake between normal surrounding pancreatic tissues during venous phase: high in normal low in tumor parenchyma

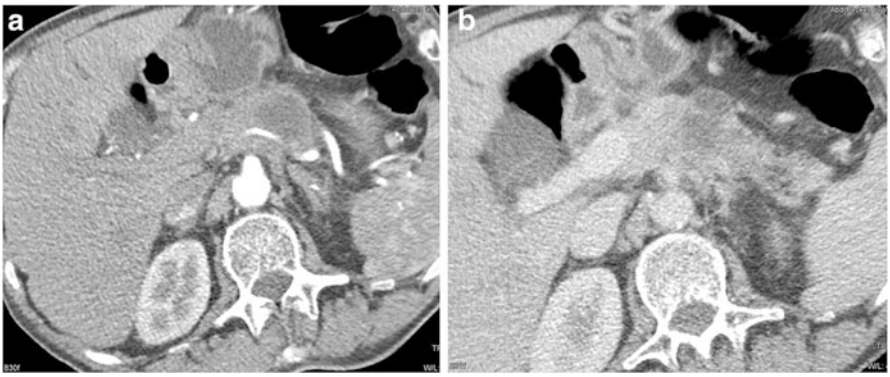

Fig. 2 T4 pancreatic adenocarcinoma of the body. The hypoattenuation sign. (**a**) Arterial phase MDCT. Large hypoattenuating focal lesion immediately left to the superior mesenteric artery with infiltration signs in the adjacent structures (mesentery, retroperitoneum, lesser sac, along-side the celiac axis). Marked hypoattenuation of the tumor tissue compared to normal surrounding pancreatic tail tissue during arterial phase. (**b**) Venous phase MDCT. Deep tumorous infiltration signs into the retroperitoneum, lesser sac, occlusion of the splenic vein, broad contact of the tumor with the celiac axis. Tumor parenchyma markedly hypoattenuating

the most recent studies shows to overpass the initial range of around 88–90% to detection rates higher than 95% [7–11]. The improved spatial and temporal resolution of these modern scanners allows the acquisition of images with a vascular enhancement ideal for tumor delineation and differential diagnosis. Current MDCT protocols utilize dual-phase techniques based on bolus tracking software to optimize

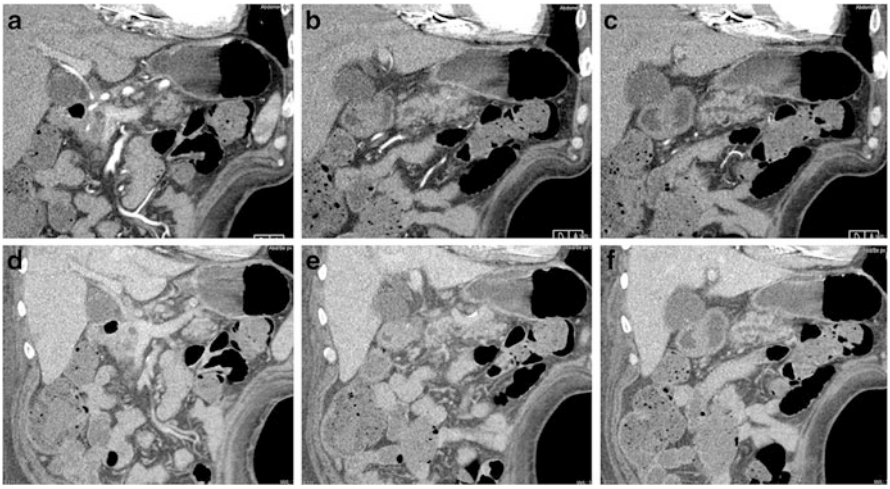

Fig. 3 T1/2 pancreatic adenocarcinoma of the head. The ductal occlusion sign. (**a**) Arterial phase MDCT axial plane at tumor level: a faintly visible tumor of the pancreatic head of around 18 mm in diameter immediately right lateral to the superior mesenteric vein. Cranial to the only minimally attenuating tumor abrupt dilatation of the pancreatic duct; (**b**) Arterial phase MDCT axial plane 10 mm more cranial than (**a**): focal dilatation of the pancreatic duct to around 10 mm; (**c**) arterial phase MDCT axial plane 20 mm more cranial than (**a**): dilatation of the pancreatic duct to around 10 mm extending to the tail; (**d**) venous phase MDCT axial plane at the same level as (**a**): as in (**a**), tumor of the pancreatic head only faintly visible without infiltrating signs to the superior mesenteric vein. Abrupt dilatation of the pancreatic duct well-demarcated; (**e**) venous phase MDCT axial plane at the same level as (**b**): focal dilatation of the pancreatic duct to around 10 mm; (**f**) venous phase MDCT axial plane at the same level as (**c**): dilatation of the pancreatic duct to around 10 mm extending to the tail

the dynamic scan parameters and acquisition following intravenous high flow injection of contrast material (4–6 ml/s, nonionic iodine dye), with depiction of the pancreas at an arterial phase (15–30s) and at a portal venous phase, respectively (40–60s). Arterial phase images are used for detection of the primary pancreatic tumor, optimal evaluation of the arterial abdominal vasculature and its relationship with the tumor, and CT angiographic delineation of vascular pathologies for staging and surgical planning (see below). Moreover, arterial phase images allow pancreatic adenocarcinoma to be distinguished from pancreatic neuroendocrine tumors, which are classically hypervascular and well enhancing in the arterial phase [7].

Particularly because of the above-mentioned characteristics, its widespread availability, its high grade of standardization CT has assumed a leading role in the diagnosis (and staging, see below) of pancreatic adenocarcinoma [7, 12, 13]. Lesion conspicuity and the discrimination between normal and tumorous tissue as contributed by MDCT are largely based on the so-called attenuation pattern during distinct phases of contrast material uptake after intravenous injection (Figs. 1 and 2). Typically, pancreatic adenocarcinoma presents as a hypoattenuating mass within the pancreatic tissue or outside when involving adjacent structures either in the

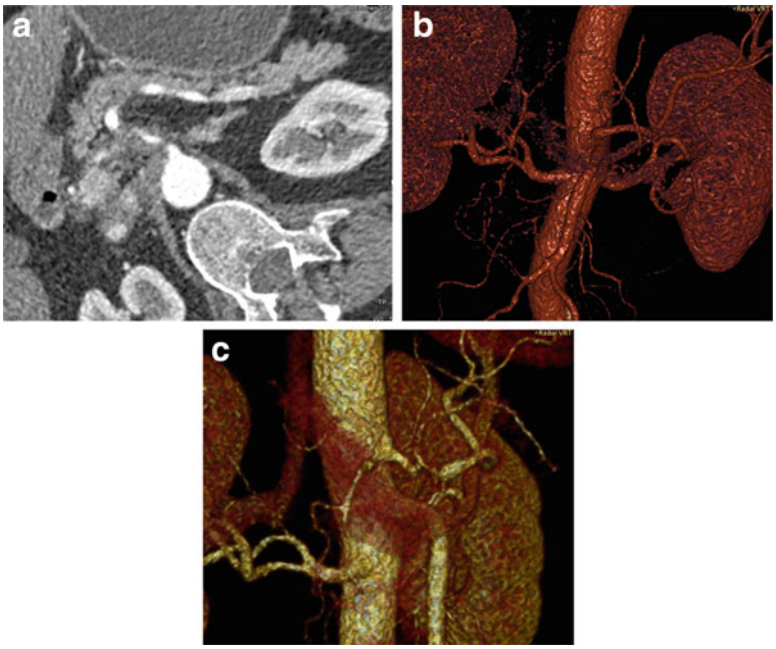

Fig. 4 Autoimmune peripancreatic vasculitis of the celiac axis mimicking infiltration by pancreatic adenocarcinoma. (**a**) Axial plane at the level of the celiac axis showing abutment and encasement of the celiac trunk and short segment occlusion of the common hepatic artery; (**b**) 3D reconstruction of the arterial phase confirming occlusion of the common hepatic artery in ap projection; (**c**) 3D reconstruction of the arterial phase confirming occlusion of the common hepatic artery in magnified right oblique projection

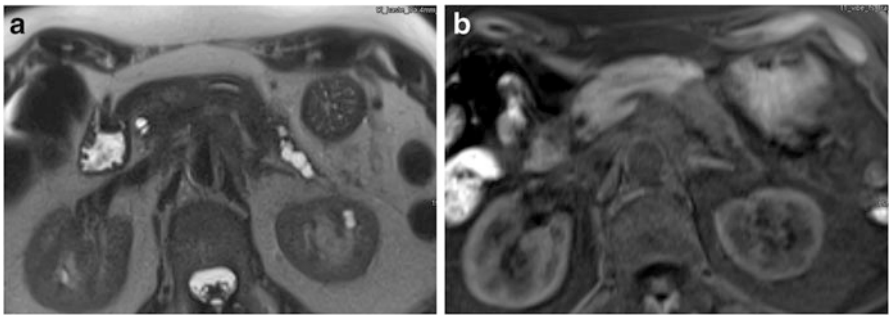

Fig. 5 MRI of a small indeterminate lesion. (**a**) Axial plane in a HASTE sequence (special high contrast and high spatial resolution water-weighted sequence): abrupt duct occlusion secondary to a small lesion of the tail of the pancreas; (**b**) axial plane noncontrast T1 gradient echo sequence showing a very high parenchymal contrast difference between tumors and normal pancreatic tissue

arterial phase after intravenous iodinated contrast material injection or at later phases, e.g., in the parenchymal (= portal venous) phase (Figs. 1 and 2). This hypoattenuation results from the significant pathologic changes in the stromal microenvironment of pancreatic adenocarcinoma versus normal pancreatic tissue. Normal pancreatic tissue displays a well-defined rise in density (= attenuation) after intravenous injection of iodinated contrast material in MDCT as expressed by the respective rise of measured Hounsfield units throughout the arterial until the parenchymal (venous) phase. Similarly, it shows a well-defined decline in density throughout the "wash-out" phase when an equilibrium of the circulating iodine contrast material in the body is reached. Such behavior at CT imaging is the direct surrogate of parenchymal vascularity reflecting an orderly and characteristic tissue structure given for each of the human organs. The microenvironment of pancreatic adenocarcinoma is widely different. Early-on, with the rising use of CT imaging (or MRI), it was noted that the tumorous tissue has a much more reduced vascular density as compared to normal pancreatic tissue [4]. Such was explained by a specific desmoplastic reaction of pancreatic adenocarcinoma. Applying anatomic imaging by MDCT (or MRI), this desmoplasia is the surrogate for the characteristic hypoattenuation at imaging. Ever since, a great amount of research has been directed toward the understanding of such specific tumor behavior as it was hoped to identify new strategy lines targeting at these microenvironmental characteristics: almost two decades ago, it was found that significant changes in gene expression are involved in the stromal desmoplasia [14]. Myofibroblasts have been described as the effector cells [15]. Cohen identified the fibroblast activation protein (FAP) as one of the major molecular pathways [16] in the pathologic tumorous microenvironment and which, furthermore, is more expressed in more advanced stages. Also, he found that the highest expression (= tissue concentration) was at the borderline between tumor and parenchyma, which is the direct reflection of imaging findings at MDCT [3–5, 17–19]. Recently, Neuzillet elucidated the counterintuitive role of SPARC (= secreted protein acid and rich in cystein) which is overexpressed in pancreatic adenocarcinoma and which, however, assumes a progression suppressing function in other solid tumors (e.g, colorectal, ovarian, prostate, breast, melanoma, and glioblastoma). The apparent contradictory function of SPARC in pancreatic adenocarcinoma, presumably, is based on the inhibition of angiogenesis via inhibition of vascular endothelial growth factor (VEGF), while promoting epithelial-to-mesenchymal transition and invasion through matrix metalloprotease expression [20]. This was supported very recently by Patsouras who described a much higher molecular concentration of FAP vs VEGF in tumor tissue from patients with IIb stages versus patients with IIa stages [21]. These findings corroborate and explain imaging characteristics at MDCT of tumor invasion into vessels, neural structures, and other surrounding tissue during the local progression of pancreatic adenocarcinoma. Hence, it might be understood now, why vascular invasion in general and into venous structures, in particular, is seen at MDCT at already relatively small tumor sizes. For example, mesenteric or portal venous invasion and obstruction are typical findings in locally advanced pancreatic adenocarcinoma and are essential features for tumor detection and differential diagnosis. Despite all these advances over the

last decade, diagnosis of small tumors (< 2 cm in diameter) is still challenging: large pancreatic adenocarcinomas are easy to identify. As described above and shown in Fig. 2, they are usually hypoattenuating (hypodense) with ill-defined margins and tend to infiltrate posteriorly into the retroperitoneum and the adjacent vessel, preferably the mesenteric vein. Besides the molecular and cellular mechanism of invasion, such infiltrating capacity is furthermore promoted by the lack of a natural barrier between the retroperitoneum and the pancreas as it has no organ capsule. Therefore, even moderate size adenocarcinoma might infiltrate into the adjacent fat, involve the common bile duct when arising in the head, and might obstruct the pancreatic duct. In small tumors (e.g., 1–2 cm), these signs of tumor spread might not be detected at MDCT and, furthermore, isoattenuation is more frequent [22]. Such isoattenuation on both arterial and venous phase images might be found in 5–10% [7, 22]. Therefore, secondary signs of a mass must be identified, including pancreatic ductal dilatation, biliary ductal dilatation, abrupt cutoff of the pancreatic duct/common bile duct at the level of the mass (Fig. 3), an abnormal contour of the pancreas, and upstream pancreatic atrophy toward the tail [23]. Moreover, the use of thin collimation or primary reconstruction (1–1.5 mm) technique allows small lesions to be better visualized. Modern scanners, nowadays, provide a host of secondary reformatting and reconstruction possibilities of the primary data set: e.g., multiplanar 2D reconstructions or 3D reconstruction techniques. The latter has gained prominence for its ability to illustrate vascular involvement. Already in 2006, the John's Hopkins group described the routine use of multiplanar reformatting techniques and 3D reconstructions to be critical in identifying small primary tumors [7, 24]: they found the use of 3D technique most important for detailed visualization of the junction of the common bile duct and pancreatic duct and for visualizing small tumors in this location. All this applies with a high level of confidence to many aspects of differential diagnosis: Peripancreatic lymph nodes, duodenal cancers, duodenal diverticula, pancreatic anatomic anomalies (such as an annular pancreas), exophytic gastric masses, and primary retroperitoneal masses have all been confused with pancreatic adenocarcinoma on axial images. As a result, modern standardized reporting protocols in imaging of pancreatic adenocarcinoma are based on these considerations and include sophisticated reconstruction techniques [12].

One area where CT finds its limits, however, is in the differentiation of pancreatic adenocarcinoma from some cases of focal pancreatitis. Particularly, reoccurring pancreatitis can result in the appearance of a focal mass, e.g., as pseudotumor, often with pancreatic and biliary ductal obstruction, which can very much mimic the appearance of a ductal adenocarcinoma. Usually, however, abrupt occlusion of the pancreatic duct at the level of a mass is more suggestive of a malignancy, and other stigmata of chronic pancreatitis in the remainder of the gland (beaded, irregular, dilated pancreatic duct, and pancreatic parenchymal calcifications) can be suggestive of focal pancreatitis. Nevertheless, infrequently reoccuring (focal) pancreatitis and especially when the duodenal groove is involved tumor mimicry can be such that a mass in the pancreatic head might be indistinguishable between benign and malignant. Even the so-called double-duct sign should and can not be used for

differential diagnosis. Another example of tumor mimicry represents autoimmune pancreatitis when presenting as a focal mass in Type 2 and not as the relative typical sausage-like appearance [25, 26]. In both instances, a careful and thorough evaluation of the given patient's history and depiction of specific serum features associated with autoimmune pancreatitis (immunoglobulin G4 levels) might be more helpful than imaging alone (see also below at the end of this chapter). Figure 4 shows an example of autoimmune vasculitis of the celiac axis, which was mistakenly interpreted first as infiltrating pancreatic adenocarcinoma, interestingly corroborated by the underlying clinical disease pattern (back pain with somewhat sudden onset). After adequate cortisone treatment complete resolution of symptoms and morphologic findings was noted, even the patency of the previously "infiltrated" and occluded common hepatic artery was restored.

In addition to MDCT and PET-CT, MRI has become an increasingly viable option in pancreatic imaging over the last decade, and each has an important role to play: In contrast to PET MRI, probably has a greater value in primary imaging and tumor detection and differential diagnosis, particularly when a suspected pancreatic lesion is not identified at MDCT. Tiny pancreatic lesions might be more conspicuous at MRI (Fig. 5) because of its superior soft-tissue contrast [22]. In cystic pancreatic lesions, its superiority in delineation and differential diagnosis is well established [7], which is being discussed in another chapter of this book. Moreover, given the limitations of MDCT in characterizing small metastatic lesions in the liver less than 1 cm, MRI is a valuable problem-solving tool when indeterminate liver lesions are detected at MDCT. Again, this underscores its potential relevance for a complete staging protocol for presurgical workup. Similarly, PET-CT has quickly become an important test to perform in conjunction with contrast-enhanced CT for the staging of a known tumor (see below).

Unlike CT and MRI, which are anatomic imaging techniques, fluoro-2-deoxy-D-glucose (FDG) PET is a functional imaging modality that uses the radiotracer 18F-FDG. This radiotracer, a glucose analog that acts as a marker for glucose metabolism, is taken up by various tissues (cells) in the body proportional to their metabolic activity, e.g., brain, heart, kidneys. Accordingly, solid tumor with a high rate of glucose metabolism will show a significant 18F-FDG uptake, usually, much higher than surrounding tissues. Like other nuclear medicine techniques, PET studies have poor spatial resolution, making it difficult to localize sites of abnormal radiotracer uptake. As a result, most studies are now performed as integrated PET-CTs, where simultaneously acquired and overlayed coregistered CT images allow accurate localization of organ involvement sites of radiotracer uptake. There is very little debate that PET-CT (especially when performed without an associated contrast-enhanced CT) should not be used as a primary imaging modality for pancreatic adenocarcinoma. The sensitivity of PET-CT (with a noncontrast, nondiagnostic CT) is considerably lower than that of a contrast-enhanced MDCT, with a sensitivity of only 72% [27]. Moreover, even in very recent and modern PET-CT scanners, the CT part does not provide the essential high spatial MDCT ($\geq$ 256 detector rows and or dual source) equipment necessary for high-quality primary imaging as outlined above. Despite that it suffers from the inability to resolve small lesions, it

is invaluable in identifying distant metastatic disease and has been shown to change the preoperative staging and determination of resectability in a sizeable number of patients (see below). Furthermore PET-CT, however, might assume a much higher relevance for the follow-up imaging of postresection patients (see below) or in distinguishing between chronically recurrent pancreatitis and pancreatic adenocarcinoma [25]. Moreover, many patients with autoimmune pancreatitis will undergo PET-CT because of overlapping symptoms with pancreatic adenocarcinoma. However, even PET-CT cannot always differentiate between these two lesions because (autoimmune) inflammatory foci in the pancreas might also accumulate FDG to the same level as compared to pancreatic adenocarcinoma [25]. Therefore, when FDG accumulation in autoimmune pancreatitis is focal, differentiation from pancreatic cancer can be difficult. Ozaki showed FDG uptake in all of their patients with autoimmune pancreatitis but only in 73.1% of their patients with pancreatic adenocarcinoma [28]. The true morphologic uptake pattern, however, of FDG-PET might be helpful for establishing a differential diagnosis. In autoimmune pancreatitis, typically, a heterogeneous longitudinal accumulation and multiple localizations are seen, whereas in pancreatic adenocarcinoma uptake is characterized by nodular homogeneous accumulation, and, of course, solitary localization [25].

Another pancreatic neoplasm entity where MDCT finds its diagnostic limits is the differential diagnosis of cystic tumors of the pancreas. In the modern radiologic literature, there is widespread consent that MRI is far superior to any other imaging modality besides, perhaps, endoscopic ultrasound [13].

Staging

Any imaging modality for staging pancreatic adenocarcinoma is applied to characterize the patient's potential for curative resection (Figs. 6, 7 and 8). Therefore, it is of common acceptance to stage patients into having (a) resectable disease (Fig. 6), (b) borderline resectable disease (Fig. 7), (c) locally unresectable disease (Fig. 8), and (d) metastatic disease including peritoneal spread and distant organ involvement.

The American National Comprehensive Cancer Network (NCCN) has been deeply involved in this definition process. The respective NCCN guidelines, version 1.2013, have been endorsed by the International Study Group of Pancreatic Surgery and were published recently [29]. They are summarized in Table 1. Furthermore, in the 8th edition of the TNM classification system [30], tumor size now plays an intrinsic role for subtyping of T1 stages (<2 cm) as follows:

T1a = tumor size $\leq$0.5 cm in maximum dimension
T1b = tumor size 0.5–1 cm in maximum dimension
T1c = tumor size 1–2 cm in maximum dimension

Furthermore, size again plays a definitive role in discrimination between T2 stage (2–4 cm) and T3 stage (>4 cm) in the tumor's maximum dimension.

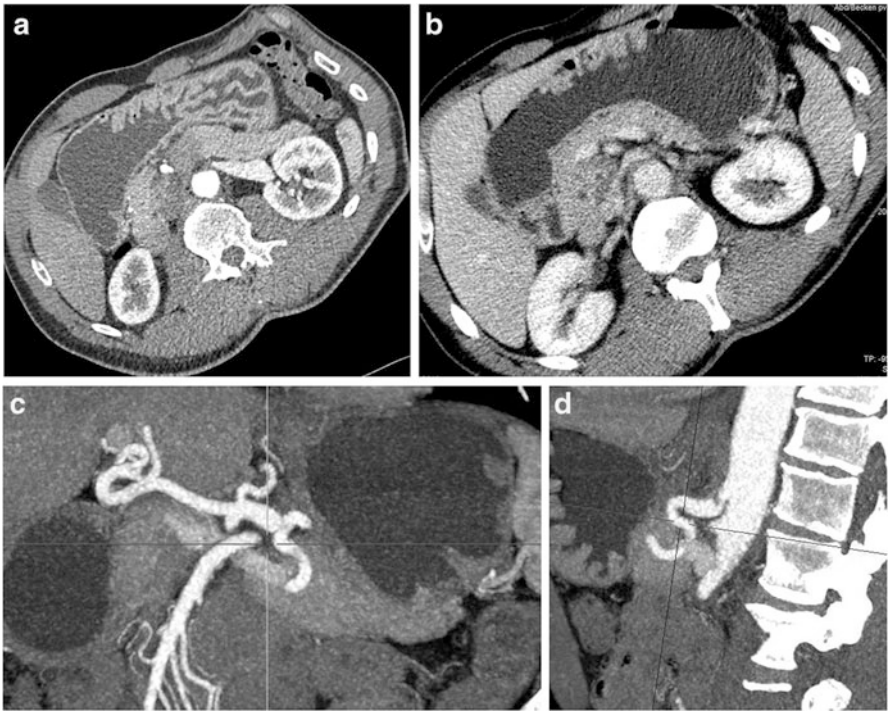

Fig. 6 Surgically resectable T2 pancreatic adenocarcinoma. (**a**) Axial plane in arterial phase at the tumor level: 2.5 cm in diameter measuring hypoattenuating mass in the dorsal aspect of the pancreatic head. (**b**) Axial plane in venous phase at the tumor level: no signs of infiltrative spread towards the superior mesenteric artery or to the superior mesenteric vein. (**c**) Coronal MIP reconstruction demonstrating complete arterial integrity. (**d**) Sagittal reconstruction demonstration potentially critical stenosis of the celiac trunk

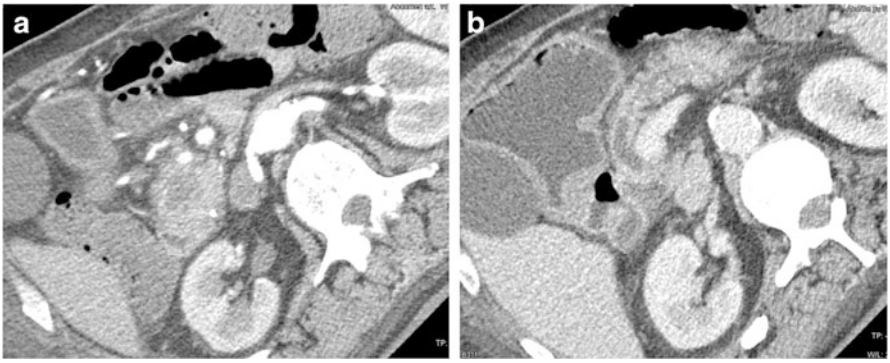

Fig. 7 borderline resectable T3 pancreatic adenocarcinoma of the head. (**a**) Axial plane in arterial phase at the tumor level showing an around 3 cm in diameter measuring tumor in the pancreatic head encasing the superior mesenteric vein by almost 360°. (**b**) Axial plane in venous phase cranial to the tumor level showing a fully open portal vein and the dilated pancreatic duct

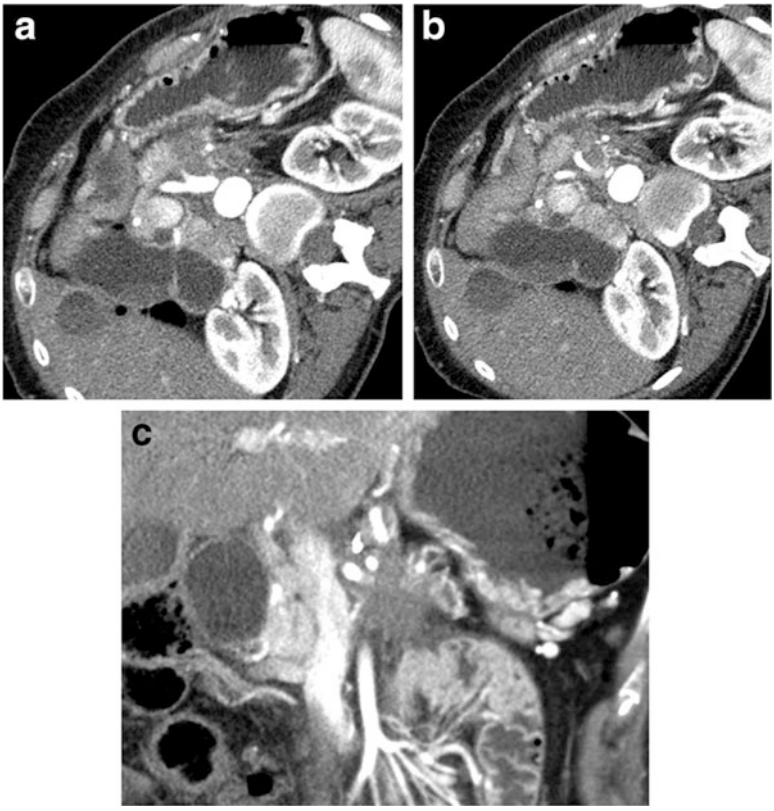

Fig. 8 Unresectable T4 pancreatic adenocarcinoma of the body. (**a**) Axial plane in arterial phase at the level of the celiac trunk showing complete encasement and hazy peritruncular hypoattenuation; (**b**) axial plane in arterial phase around 1 cm more cranial than (**a**) showing complete encasement and hazy peritruncular hypoattenuation of both the common hepatic and the splenic artery; (**c**) coronal reconstruction in arterial phase to illustrate the complete encasement of the celiac axis

In particular, this latest version of the TNM classification system [30] pays tribute to the now widely accepted fact that size is the most important prognostic factor in pancreatic adenocarcinoma alone. This relationship between tumor size and survival has been established very early [31] and was corroborated during the ongoing history of pancreatic surgery in many studies [31–36].

Moreover, as a result of a very recent large retrospective study of the correlation between tumor size and survival after curative resection, especially of T3 pancreatic adenocarcinoma, Kurata suggests a further subdivision of T3 stages by applying a tumor diameter of 3 cm at imaging as cut off to discriminate between the so-called T3a and T3b stages. In his study of 755 resected patients with a T3 stage, patients with a tumor smaller as T3 ($n = 274$) had a median survival time of 30.3 months. Patients with resected tumors larger than 3 cm in maximum diameter had a median survival time of 17.5 months. This large influence on tumor-free survival was highly

statistically significant: in a univariant analysis, the p-value was 0.002; and in a multivariant analysis, it was 0.005, respectively [37]. However, it remains to be seen, whether such will find relevance for prospective revisions of the TNM classification system. Nevertheless, as a routine, the correct description of tumor dimensions in all planes is part of the routine imaging process [3, 5, 7, 12, 13, 38, 39].

When putting all these facts and study results into perspective for a modern presurgical workup, it becomes evident that adequate (= high resolution) imaging is the key for addressing patients' hopes for a potentially curative procedure or avoiding unsuccessful resection (Figs. 6, 7, and 8).

As illustrated and detailed in Table 1, determining vascular involvement is the most important component of determining resectability of pancreatic adenocarcinoma. As it has been stated before, modern state-of-the-art MDCT, definitely, plays the most important role. Hence, structurized and standardized reporting tools and templates have been developed and employed to help radiologists and surgeons to communicate adequately on all the essential details of imaging for resectability. A reporting template [12] was developed as a result of a consensus conference during the annual American Pancreatic Association meeting (Chicago 2011) based on earlier work [18, 39].

However, several facts are important to note, though, which are pertinent for gaining the entire perspective of resectability of pancreatic adenocarcinoma and are not reflected in the TNM classification specifically or in the guidelines as summarized in Table 1: (1) Arterial anatomic variants are very frequent. E.g., an aberrant branch from the SMA to the right liver lobe is not uncommon (up to 15%) and might hamper head resection. (2) Stenosis of the celiac trunk either from atherosclerosis or from the left diaphragm tendon is a relatively frequent finding in the elderly which

Table 1 NCCN guidelines on resectabilty of pancreatic adenocarcinoma, version 1.2013 [29], summarized for the pancreatic head

Localized and resectable	Borderline resectable	Unresectable
No distant metastasis	No distant metastasis	Distant metastasis
No radiographic evidence of SMV or PV distortion	Venous involvement of the SMV or PV with distortion or narrowing of the vein with suitable vessel proximal and distal, allowing for safe resection and replacement	Greater than 180° SMA encasement, any celiac abutment, IVC infiltration
Clear fat planes around CA, HA, and SMA	GA encasement up to the hepatic artery with either short segment encasement or direct abutment of the HA without extension to the CA	Unreconstructable SMV/portal vein occlusion
	Tumor abutment of the SMA not to exceed 180° of the circumference of the vessel wall	Aortic invasion or encasement

CA celiac axis, *GA* gastroduodenal artery, *HA* hepatic artery, *IVC* inferior vena cava, *PV* portal vein, *SMA* superior mesenteric artery, *SMV* superior mesenteric vein

should be known before dissecting the gastroduodenal artery and thereby avoiding liver hypoxia (Fig. 6c). (3) Anatomic variable course of the inferior mesenteric vein when not draining into the splenic vein. (4) Involvement of the common hepatic artery close to the liver hilum. Moreover, for adenocarcinoma located in the tail of the pancreas or at least distinctly left of the superior mesenteric different resection strategies might apply.

Therefore, state-of-the-art imaging for staging needs to address exact localization of the tumor, exact determination of its size in all dimensions, arterial and venous vascular mapping (as described above and Table 3), thorough analysis of fatty tissue and perivascular involvement (at CT hazy hyperattenuation of fatty tissue planes = stranding) or eventual vascular abutments and lack of anatomical integrity, exclusion of liver metastasis or to other distant organs. Based on such state-of-the-art imaging, the standardized reporting system has to address and describe all details relevant for resections strategies regardless of how borderline surgical resectability is defined [40]. In Table 2, our general recommendations for CT scanning are summarized. Table 3a, b describe our suggestions for the reporting standards for arterial and venous involvement including a description of anatomical variants. These are unanimously valid and can be universally applied notwithstanding the still existent differences at present in the definition of borderline resectable pancreatic adenocarcinoma [29] across various institutions and cancer centers, which was recently analyzed in depth by Pietryga (see Table 2 in his publication) [40].

Table 2 CT imaging strategies for detection and staging of pancreatic adenocarcinoma

CT: Technical details	Imaging purpose
Helical scan type	High resolution axial planes, secondary 2D and 3D reconstructions
1 mm or submillimeter scans	Optimized spatial and contrast resolution, 2–3 mm image reconstructions
1 mm reconstruction intervals	Optimized spatial and contrast resolution, 2–3 mm image reconstructions
Pitch factor 10–15	Fast scanning compromising between homogeneous contrast phase imaging and background noise
20–30° left oblique patient's positioning + oral warm water (0.5 l) as negative contrast agent	Optimized duodenal filling and dilatation for best delineation of ampullary region
100–125 ml i.v. iodine contrast agent (≥300 mg iodine/ml, non-ionic)	Arterial and venous phase imaging, preferably using automatic dynamic scanning (better than fixed delays)
General imaging aspects	
Morphologic evaluation of the tumor location in relationship with the three anatomic pancreatic regions (head/uncinate, body, tail)	
Hypo, iso, or hyperattenuating appearance in the three contrast phase acquisitions	
Bile duct appearance, related or not to the tumor location	
Pancreatic duct appearance, related or not to the tumor location	
Pancreatic parenchymal appearance "upstream" to tumor location	
Parenchymal calcifications, cysts	

Table 3 Evaluation of vascular invasion patterns

Anatomic details vessel type	Imaging findings confirmation/exclusion
a) Arterial vessel analysis	
General abdominal vessel anatomy	Absence or presence of variant arterial vascular anatomy: e.g., narrowing of the celiac trunk, right aberrant hepatic artery originating in the superior mesenteric artery
Celiac trunk	Normal course. Presence or absence of stenosis or variants. Presence or absence of solid tumor contact. Presence or absence of perivascular infiltration (stranding, hazy attenuation increase peritruncular). When present: $\geq 180^\circ$ of vessel circumference or less. Presence or absence of contour irregularity
Common hepatic artery	Normal course. Presence or absence of stenosis or variants. Presence or absence of solid tumor contact. Presence or absence of perivascular infiltration (stranding, hazy attenuation increase periarterial). When present: $\geq 180^\circ$ of vessel circumference or less, extension to celiac trunk, extension to right or left hepatic artery. Presence or absence of contour irregularity
Superior mesenteric artery	Normal course. Presence or absence of stenosis or variants. Presence or absence of solid tumor contact. Presence or absence of perivascular infiltration (stranding, hazy attenuation increase periarterial). When present: $\geq 180^\circ$ of vessel circumference or less, extension to celiac trunk, extension along main stem (branch involvement). Presence or absence of contour irregularity
Splenic artery	Normal course. Presence or absence of stenosis or variants. Presence or absence of solid tumor contact. Presence or absence of perivascular infiltration (stranding, hazy attenuation increase periarterial). When present: $\geq 180^\circ$ of vessel circumference or less, extension to celiac trunk, extension along main stem. Presence or absence of contour irregularity
b) Venous vessel analysis	
General abdominal vessel anatomy	Absence or presence of variant venous vascular anatomy: e.g., aberrant drainage of inferior mesenteric vein into main stem of superior mesenteric vein. Normal course of inferior vena cava. Presence or absence of venous collaterals/varices
Portal vein	Normal course. Presence or absence of stenosis or variants. Presence or absence of solid tumor contact. Presence or absence of perivascular infiltration (stranding, hazy attenuation increase perivenous). When present: $\geq 180^\circ$ of vessel circumference or less. Presence or absence of contour irregularity. Presence or absence of thrombus/occlusion. When present: length of thrombus/occlusion
Superior mesenteric vein	Normal course. Presence or absence of stenosis or variants. Presence or absence of solid tumor contact. Presence or absence of perivascular infiltration (stranding, hazy attenuation increase perivenous). When present: $\geq 180^\circ$ of vessel circumference or less. Presence or absence of contour irregularity. Presence or absence of thrombus/occlusion. When present: length of thrombus/occlusion, involvement of distal draining vessels (e.g., aberrant inferior mesentic vein, jejunal branches)

(*continued*)

Table 3 (continued)

Anatomic details vessel type	Imaging findings confirmation/exclusion
Inferior vena cava	Normal course. Presence or absence of stenosis or variants. Presence or absence of solid tumor contact. Presence or absence of perivascular infiltration (stranding, hazy attenuation increase perivenous). When present: $\geq 180^{\circ}$ of vessel circumference or less. Presence or absence of contour irregularity. Presence or absence of thrombus/occlusion. When present: length of thrombus/occlusion
Splenic vein	Normal course. Presence or absence of stenosis or variants. Presence or absence of solid tumor contact. Presence or absence of perivascular infiltration (stranding, hazy attenuation increase perivenous). When present: $\geq 180^{\circ}$ of vessel circumference or less. Presence or absence of contour irregularity. Presence or absence of thrombus/occlusion. When present: length of thrombus/occlusion

As stated already above, MRI has advantages in delineating small tumors because of better lesion conspicuity when applying the inherent various tissue characterization parameters in combination with diffusion-weighted imaging (DWI), which is superior to the solely anatomic imaging character of MDCT. Moreover, its higher accuracy in differentiating liver lesions attributable also to its higher soft tissue characterization potential might be used for a more complete and correct staging as compared to MDCT alone. MRI is much better at defining hepatic lesions and can characterize small hemangiomas or cysts as definitively benign, as already stated above. Metastases are typically mildly hypointense on T1-weighted images and mildly hyperintense on T2-weighted images. Containing little internal fluid, metastases can be definitively differentiated from cysts or hemangiomas by their lower T2 signal. Most lesions will show peripheral enhancement on postcontrast images, along with wedge-shaped perilesional enhancement in the arterial phase. Moreover, DWIs have been shown to be substantially more sensitive for small liver metastases compared with MDCT, as liver metastases have a significantly lower ADC value than the surrounding liver [41, 42].

PET alone is not useful in the evaluation of local resectability and locoregional staging: The poor spatial resolution of PET makes it difficult to establish the relationship of the mass relative to adjacent organs and vascular structures [43]. Moreover, high radiotracer uptake in the primary mass almost always renders subtle evaluation of the surrounding tissues near the tumor bed difficult, which in particular will make a clear definition of vascular involvement relevant for resection nearly impossible as outlined in Table 3. In addition, PET-CT has no advantage compared to regular thin slice MDCT in local lymph node staging for all of the above-mentioned reasons. The sensitivity and specificity of PET-CT for local lymph node metastases may be as low as 46% and 63%, respectively [44, 45].

Similarly, the value of PET-CT for a subtle staging work-up to rule out liver metastasis is limited: the poor resolution of PET for lesions less than 1 cm might be held responsible for this poor performance. On the other hand, PET-CT does seem to

be a very valuable adjunct to contrast-enhanced MDCT in the evaluation of distant metastatic disease, e.g., lung and bones. In particular, for bone metastasis, a sensitivity of up to 100% has been reported [46]. Hence, as a standalone examination in the evaluation of hepatic metastases, PET-CT (when performed without a diagnostic contrast-enhanced CT) has, in summary, the following and significant limitations: The sensitivity of the study for hepatic metastases overall is only about 70%, with a sensitivity for lesions less than 1 cm of only 43% (although the specificity is relatively high, ranging up to 95%) [44, 45].

Therefore, the clinical practice guidelines of the NCCN acknowledge the potential utility of PET-CT in the staging of pancreatic adenocarcinoma but state that it is not a substitute to state-of-the-art (high resolution) MDCT [47, 48].

Postsurgical Imaging, Recurrence Recognition Pattern

Imaging after surgical resection of pancreatic adenocarcinoma, usually entailing the Whipple procedure with or without preservation of the pylorus, needs to address immediate or early ruling out of postsurgical complications and, during follow-up, ruling out of recurrent disease or other late complications.

Resection of pancreatic adenocarcinoma applying the Whipple procedure was once associated with high peri- and postoperative morbidity and mortality rate [49]. However, significant improvements in surgical skills and technique and, furthermore, peri- and postoperative critical care over the last three decades have reduced the 30-day mortality rate to as low as 1% in the highest-volume centers [29, 34, 35, 50]. Even in the elderly, it can be performed with very low mortality [51].

Besides the above-cited refinements in surgical technique and increasingly sophisticated critical care, MDCT has achieved a major relevance in the early and precise identification of complications that can occur after the Whipple procedure, and it has a great potential and responsibility for directing early actions to reduce both major morbidity and mortality. Many of the below mentioned classical complications are amenable to less invasive measures applying interventional radiological procedures. These, however, are beyond the scope of this chapter.

Like in the preoperative workup schedule, standardized and refined imaging protocols are required to discriminate between normal postoperative findings and true complications, and to report or rule out correctly the host of postoperative complications that might be encountered after a Whipple procedure, including pancreatic fistula, postoperative abscesses, bile leakage, portal vein or superior mesenteric vein thrombosis, postoperative hemorrhage, pseudoaneurysm formation, ischemic hepatic complications, and during follow-up pancreatic and bile duct strictures (mostly anastomotic). Moreover, without profound knowledge of the applied surgical procedural details, not such an approach or claim can be successfully realized. Any radiologist performing post-Whipple imaging studies need to fully understand the course of preparational events and hazards during the complex surgical resection steps. Conversely, the surgical report needs to address any little detail potentially relevant for the appearance of any of the above-mentioned

complications. Today, the challenges and risks during the various steps of the Whipple procedure are well defined with respect to (a) the pancreaticojejunostomy [52–54] with its risk of leakage [55], septical complication, and hemorrhage [56]; (b) the bile duct anastomosis [54] with its risk of early leakage and delayed stricture; (c) early and late hemorrhage [56] as a result of either direct preparation, complex vascular anastomotic procedures, or occurring with a somewhat unpredictable delay as a consequence of septic erosions; (d) the gastrojejunostomy [57] regardless whether part of the classical Whipple Kausch operation removing the pylorus or the modified technique preserving the pylorus including early anastomotic leakage or delayed emptying failures; (e) portal vein or superior mesenteric vein thrombosis and arterial occlusion, respectively [58], resulting either in hepatic ischemia or delayed variceal hemorrhage.

Normal Postsurgical Morphologic Features

The timing of postsurgical MDCT study with regard to the postsurgery course largely determines the morphologic appearance of the postoperative abdominal findings and it can vary substantially (Fig. 9). Nevertheless, and regardless of whether the study is performed in the very early postoperative period or as part of the routine surveillance program, the three anastomoses of the Whipple operation must be carefully evaluated:

1. Pancreaticojejunostomy: A jejunal loop is pulled up transmesenterically and anastomosed to the right of the pancreatic remnant. Often, the pancreatic duct can be visualized from the pancreatic tail toward the anastomosis. In an early postsurgical phase, collapsed loops of bowel adjacent to the pancreaticojejunostomy might be misinterpreted as hematoma. In later follow-up imaging studies, such collapsed bowel loops can mimic tumor recurrence. Similarly, the morphologic appearance of the anastomosis can present as a "bulge" of the jejunum into the residual part of the pancreas, which again can be misinterpreted as tumor recurrence. Multiplanar reformation or 3D postprocessing can be particularly valuable in visualizing these confounding issues related to the pancreaticojejunostomy. In an acute or early postsurgery situation, the blind end of the jejunal loop might assume a hazy appearance from swelling. Its anatomic position is not much variable and should be identified somewhere behind the left liver lobe toward the remnant of the stomach in the classic Whipple Kausch operation or near the pylorojejunostomy in its modified version with a preserved pylorus.
2. Bile duct anastomosis (hepaticojejunostomy or choledochojejunostomy): For the anastomosis between the bile duct system and the intestinum, the same loop as for the pancreaticojejunostomy is used but further "downstream." The anastomotic morphology can be difficult to interpret in the axial plane. In a normal postoperative situation, usually, gas is present in the bile duct(s). Such pneumobilia can be considered as a sign of well-functioning anastomosis when gas can freely move up and down through. Most often, the gas in the intrahepatic bile ducts can be

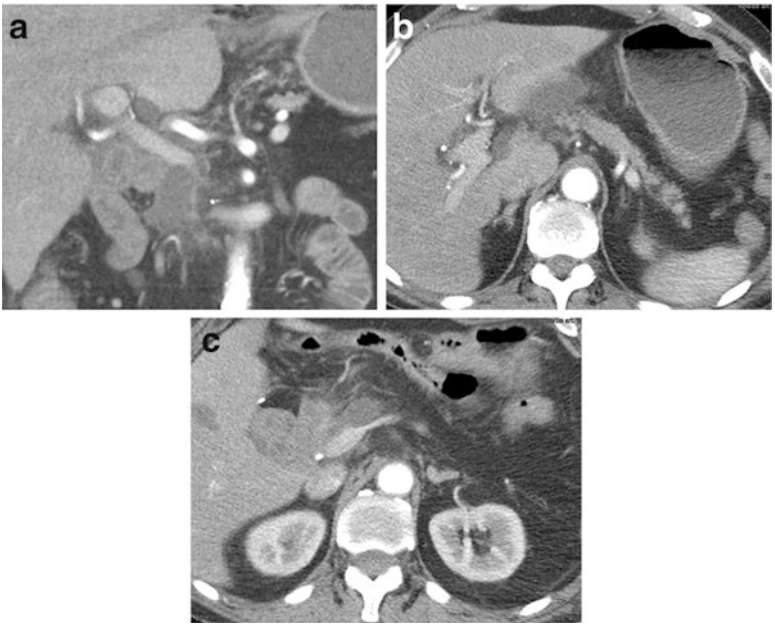

Fig. 9 Normal postoperative MDCT study day 8 after Whipple operation with preserved pylorus. (**a**) Coronal view in arterial phase (2 mm reformat): note the anatomy with a jejunal loop for both the pancreaticojejunostomy and the biliary-enteric anastomosis. The hepatic ligament shows the typical postsurgical hazy appearance resulting from the detailed preparational steps. In the surgical bed, there is a small unsuspicious fluid collection. Both anastomoses show mild swelling and hazy environments. Portal vein and proper hepatic artery are well perfused. (**b**) Axial view in arterial phase (2 mm): the pancreaticojejunostomy shows mild swelling, no signs of leakage, abscess or hemorrhage. (**c**) Axial view in arterial phase (2 mm): the hepaticojejunostomy shows mild swelling, no signs of leakage, abscess, or hemorrhage. Superior mesenteric and splenic vein well perfused. Minor local fluid collection ventral to the confluens

traced centrally toward the anastomosis. The bowel loops of the hepaticojejunostomy are rarely distended, and, like described already for the pancreaticojejunostomy, such collapsed loops of jejunum in the right upper quadrant and the near the anastomosis should not be mistaken for tumor recurrence when imaging is done during later follow-up.

3. Gastrojejunostomy: In the modified Whipple operation, the jejunum is anastomosed to the pylorus, which nowadays is mostly the case. The exact position of this anastomosis varies depending on the institution and its surgical tradition and technique. This anastomosis should be best evaluated by applying oral contrast agent. For this, warm still water and chemical intestinal spasmolysis are preferred to achieve the best morphologic situation possible.

In early postoperative imaging minor fluid collections, anastomotic edema and fat stranding in the mesentery and in the fat planes surrounding the major

abdominal vessels are common imaging features and should not be misinterpreted as a residual tumor or an abnormal inflammatory process. Often termed as induration, reflecting hazy hyperattenuation zones surrounding the superior mesenteric vein and superior mesenteric artery such reaction is very typical as a result of the detailed surgical resection steps, e.g., vessel preparation and lymph node dissection. Sometimes it can be very focal and masslike in appearance, and should not be misinterpreted as a residual tumor. However, for a correct diagnosis, full information as to the resection status is mandatory. The first postoperative imaging procedure forms the baseline for any follow-up studies, which should show resolution or stability of these immediate postoperative findings (see also below). It is also common and acceptable to visualize multiple prominent lymph nodes in the central mesentery and the sites of surgical maneuver and exploration, sometimes measuring more than 1 cm in size, and these lymph nodes are almost always reactive in the acute setting. Again, close attention should be paid to these lymph nodes on subsequent follow-up studies to ensure that they either remain stable or resolve. Because of anastomotic edema, thickening at the gastrojejunostomy is a common finding. For the same reason, dilatation of the pancreatic duct might be observed secondary to edema at the pancreaticojejunostomy. And mild intrahepatic biliary dilatation should not be misinterpreted as an early biliary-enteric stricture because this finding usually reflects mild postoperative edema at the hepaticojejunostomy and will typically resolve during the later course.

Pathologic Postsurgical Findings, Complications

Leakage of the Hepaticojejunostomy or Choledochojejunostomy

Although leakage of the biliary-enteric anastomosis of the Whipple operation is a relatively rare complication ranging from 2% in high volume centers [59, 60] to 8% in smaller series [61], it might have dreadful consequences and might further perpetrate other complications associated with high morbidity and mortality [59]. Bile leaks typically appear within the first week after surgery as bilirubin-rich drainage fluid in the surgical drains. Associated clinical signs include fever, leukocytosis, and increased CRP levels. Patients with a bile leak frequently develop other complications, including a pancreatic fistula, wound infection, delayed gastric emptying, and sepsis. A severity grading system has been proposed by the International Study Group of Liver Surgery [62]: bile leakage was defined as bilirubin concentration in the drain fluid at least three times the serum bilirubin concentration on or after postoperative day 3, or as the need for radiologic or operative intervention resulting from biliary collections or bile leakage peritonitis. The severity of bile leakage was classified according to its relevance for patients' clinical management. Grade A bile leakage does not require any change in patients' clinical management. A Grade B bile leakage requires active therapeutic intervention but is manageable without relaparotomy, whereas in Grade C, bile leakage relaparotomy or a prolonged

percutaneous transhepatic drainage is required [59, 63], which, however, can be successfully performed ever after failed surgical repair attempts [63]. As a consequence of findings in a high-volume center and revising the results of 715 operations, Burkhart proposes a slightly different grading system: Grade A bile leaks are those managed with prolonged drainage by operatively placed drains, grade B bile leaks with percutaneous abdominal drainage, and grade C bile leaks with insertion of a percutaneous transhepatic biliary drainage, respectively. Such is the routine in our institution [51].

In some patients, the diagnosis of a bile leak is suggested by the presence of a focal fluid collection or biloma close to the biliary-enteric anastomosis at MDCT. However, confirmation should be based on the above-mentioned biochemical findings.

Pancreatic Fistula

Pancreatic fistula as a result of failure of the pancreaticojejunostomy is considered as one of the most frequent causes of serious postoperative morbidity after the Whipple procedure: The reported rate of pancreatic fistula may be anywhere between 6% and 14% depending on the exact definition of a fistula as discussed below, and the mortality rate ranges from 1.4% to 3.7% [64]. In 2016, the International Study Group of Pancreatic Fistula classification has become the gold standard in defining postoperative pancreatic fistula in clinical practice. According to this, a clinically relevant postoperative pancreatic fistula is defined as a drain output of any measurable volume of fluid with an amylase level >3 times the upper limit of institutional normal serum amylase activity, associated with a clinically relevant development/condition related directly to the postoperative pancreatic fistula. Consequently, grade A postoperative pancreatic fistula is defined and called a "biochemical leak," because it has no clinical importance and is no longer referred to a true pancreatic fistula. Postoperative pancreatic fistula grades B and C are defined as follows: Grade B requires a change in the postoperative management; drains are either left in place >3 weeks or repositioned through endoscopic or percutaneous procedures. Grade C postoperative pancreatic fistula reflects those postoperative pancreatic fistulas that require reoperation or lead to single or multiple organ failures and/or mortality attributable to the pancreatic fistula [55].

Moreover, a pancreatic fistula is associated with a number of other direct or indirect complications, including pancreatitis, abscess formation, hemorrhage, delayed gastric emptying, and sepsis [64]. The development of an abscess or sepsis in conjunction with a pancreatic fistula can have a mortality rate ranging from 20% to 40% [65]. Although the drain output is the key to diagnosis, as stated before, MDCT can be helpful in identifying pancreatic fistula: The presence of a focal fluid collection or hemorrhage adjacent to the pancreaticojejunostomy is strongly suggestive, particularly if the collection appears to be in contiguity with the pancreatic duct or anastomotic suture line.

Leaks from the Gastrojejunostomy

Unlike pancreatic fistula, which is a relatively common complication of the Whipple procedure, leaks from the gastrojejunostomy are much rarer, and this complication has not been well characterized in the surgical literature. However, in a series of 3000 patients who underwent either a classic or pylorus-sparing Whipple procedure for a number of different indications, Winter found a gastrojejunostomy leak only in 0.4% [66]. When these leaks occur, though, they are associated with significant morbidity; 12 of the 13 patients in this series ultimately required surgical intervention, and four of the 13 patients died as a result of the complication. Because of the rarity of this leakage, information from the radiology literature regarding the typical imaging manifestations is very sparse. A relatively clear suspicion arises when a fluid collection directly adjacent to the gastrojejunostomy is seen. In such a rare finding, a positive oral contrast material might have an advantage over the usually applied negative oral contrast using warm water. Then, direct extravasation of the positive contrast material at the anastomosis is strongly suggestive of such leakage from the gastrojejunostomy.

Abscesses

The incidence of an intraabdominal abscess after the Whipple procedure, regardless of the underlying cause, ranges up to 6% [67]. Intraabdominal abscesses can arise secondary to an underlying pancreatic fistula, superinfection of an acute postoperative fluid collection, leakage from the hepaticojejunostomy, or leakage from the gastrojejunostomy or duodenojejunostomy, as already stated above. Hence, MDCT in early postoperative phase is most often performed to search for fluid collections suspicious for postsurgical abscesses. Diagnosis of an abscess should be based on (a) presence of an at least mildly attenuating fluid collection with Hounsfield units above 10, (b) contrast uptake of a rim-like delineation wall, and (c) adequate clinical suspicion including respective laboratory findings.

Postoperative Hemorrhage

Although not very frequent (up to 14%), postoperative hemorrhage after the Whipple procedure might present as a dreadful complication, with a mortality rate up to 40% [68, 69]. There are two distinct groups of patients based on the timely appearance. Early postoperative hemorrhage occurs within the first 24 h after surgery and often results from active bleeding from a leaking stump of the gastroduodenal artery mainly because of inadequate ligation during surgery. Late postoperative hemorrhage occurs mostly between 5 and 15 days and is usually secondary to erosions of the mesenteric vasculature as a result of inflammatory complications from leakages, perhaps around 60%, and sepsis [68, 70]. The

International Study Group of Pancreatic Surgery (ISGPS) developed an objective, generally applicable definition of postpancreatectomy hemorrhage: Postpancreatectomy hemorrhage is defined by three parameters: onset, location, and severity. The onset is either early ($<$ or $=$ 24 h after the end of the index operation) or late ($>$24 h). The location is either intraluminal or extraluminal. The severity of bleeding may be either mild or severe. Three different grades (grades A, B, and C) are defined according to the time of onset, site of bleeding, severity, and clinical impact [56]. Intraluminal hemorrhage will usually present with hematemesis or melena, whereas extraluminal hemorrhage is suspected when blood appears in abdominal drains or when hemoglobin levels are acutely dropping. Extraluminal intraabdominal hemorrhage is much more common than intraluminal hemorrhage by a ratio of 2:1 [71]. The initial presence of blood from either intraluminal or extraluminal source has been termed "sentinel" bleeding and requires urgent measures as has been nicely demonstrated in a large and very recent series of Ansari [69]. In very acute clinical settings of unstable patients, CT may not be a consideration but rather angiography and interventional radiology. When hemorrhage from the stump of the gastroduodenal artery then is detected, immediate surgery is preferred, as interventional measures might become very cumbersome and time-consuming, such as trying to implant stent grafts to seal off the bleeding stump. Placing a small blocking balloon catheter into the celiac axis may be very helpful under such conditions. In patients who are stable, MDCT is extremely helpful. Arterial phase imaging should identify the active extravasation sites or pseudoaneurysm formation. Besides adequate phase, correct timing of intravenous contrast medium injection nor further preparational steps should be applied in the interest of time.

Postoperative Pancreatitis

The presence of postoperative pancreatitis can have significant prognostic implications, including a higher risk of pancreatic fistula and, for poorly understood reasons, a higher risk of delayed gastric emptying (see below). As fat stranding and inflammatory changes in the mesentery, around the major abdominal vessels and surrounding the residual pancreatic tissue are common findings, differentiating pancreatitis from normal postoperative inflammation can be difficult, particularly in cases of mild pancreatitis. The true incidence of post-Whipple pancreatitis is unknown, but given that CT can detect only severe cases, the incidence is likely higher than the 27% (10/37) reported by Räty [72]. Nevertheless, in severe cases, MDCT confirms the diagnosis of postoperative pancreatitis when severe peripancreatic inflammatory changes, low attenuation fluid collection with direct contact with the pancreatic remnant, and hypoattenuating fatty infiltration are present. Moreover, a disproportionate amount of fluid in the pararenal spaces can be another clue suggesting pancreatitis [58].

Portal Vein and Superior Mesenteric Vein Thrombosis

During the last 10 years, a new category of borderline resectable tumors has emerged, including tumors that involve $\leq 180^{\circ}$ of the circumference of the superior mesenteric artery, abut or encase the hepatic artery for a short segment, or narrow or occlude the superior mesenteric or portal vein for a short segment. Adequate surgical options now exist for vascular reconstruction for patients with such conditions, as summarized in Table 1 and resulting from several consensus initiatives [29, 38, 73, 74]. This is also reflected, to some extent, in the latest (8th) edition of the TNM classification [30]. As a result, the complexity and incidence of surgical venous reconstructions have markedly increased, and it is not rare for patients to undergo venous resections with either primary anastomosis or the insertion of a venous interposition graft even in the elderly [51]. The development of mesenteric venous thrombosis can have disastrous consequences, including intestinal ischemia, uncontrolled ascites, hepatic ischemia, and death [75, 76]. Thererfore, for the diagnosis of superior mesenteric and/or portal vein thrombosis, MDCT needs to be carried out very carefully with special attention to the adequate timing of the intravenous contrast material. Eventually, special venous phase imaging with automated dynamic scanning might become necessary. The coronal reconstructions as 2D or MIP projections are very important for accurate diagnosis, particularly for thrombus detection in the superior mesenteric vein. The latter should be carefully evaluated along its course to rule out short-segment filling defects which can be difficult to visualize on the axial source images.

Hepatic Infarction

At postsurgical MDCT hepatic infarction is relatively ease to detect or rule out, provided that adequate phase correct dynamic imaging is performed. The underlying arterial occlusion should be detected similarly to the previously mentioned venous thrombosis on high-quality coronal images, including 2D and MIP projection. Infarcted hepatic tissue, usually, presents as a demarcated and zonal hypoattenuation parenchymal area both in arterial and venous phases. It is a relatively rare complication because of dual blood supply to the liver. Nevertheless, it is a well-known complication of the Whipple procedure. Most patients have an underlying abnormality in their mesenteric arterial vasculature, eventually overlooked at imaging for primary staging (see above), or as a result of severe atherosclerotic disease, median arcuate ligament syndrome, fibromuscular dysplasia, or previously unknown mesenteric vasculitis. These patients are uniquely vulnerable to postoperative variations and decreases in blood flow as a result of hypotension, sepsis, and more [77]. Another surgical complication that can overlook on the preoperative imaging or at the time of surgery [77]. The consequences of hepatic infarction can be severe, with a mortality rate approaching 50% after hepatobiliary surgery. In cases of common (or proper) hepatic artery injury or thrombosis, the most common result is infarction

of the left hepatic lobe, with subsequent development of biliary necrosis, hepatic superinfection, and hepatic abscesses.

Delayed Gastric Emptying

A common complication of the Whipple procedure, the exact incidence of delayed gastric emptying is unclear, largely because of the lack of a consensus definition [57]. Depending on the exact definition used, the incidence may be as high as 49% [57]. The International Study Group of Pancreatic Surgery (ISGPS) developed an objective and generally applicable definition with grades of delayed gastric emptying based primarily on severity and clinical impact. Three different grades (A, B, and C) were defined based on the impact on the clinical course and on postoperative management [57]. Although not a diagnosis primarily as result of imaging, the presence of a severely distended stomach filled with oral contrast material can be highly suggestive in the suspected clinical setting. Fluoroscopic oral contrast studies of gastric emptying rather than CT alone may be helpful in better confirming this diagnosis. Clinically, delayed gastric emptying is often diagnosed on the basis of a persistent need for a nasogastric tube after surgery or the need to reinsert a nasogastric tube several days after surgery. The exact cause of this complication is unknown but is likely related to localized disturbance of the autonomic innervation of the stomach near the operative bed. Interestingly, the likelihood of delayed gastric emptying is thought to be perpetrated by other previous postsurgical complications, including the development of an abscess, pancreatic fistula, and severe intraoperative blood loss [78].

Late Complications

Anastomotic Strictures

Anastomotic strictures can be identified at both the pancreaticojejunostomy and hepaticojejunostomy. Patients with biliary strictures present with cholangitis and jaundice, whereas patients with pancreaticojejunostomy strictures present with diarrhea, steatorrhea, abdominal pain, and pancreatic insufficiency. CT has a valuable role in the diagnosis of anastomotic strictures at these two sites because any change in the size of the pancreatic duct or intrahepatic bile ducts should be looked on with suspicion. However, change in duct size should result in a careful evaluation of the anastomotic site for any signs of local tumor recurrence resulting in ductal obstruction.

Tumor Recurrence

Although the vast majority of patients with disease recurrence present with distant metastatic disease, up to 40% of patients present with isolated local recurrence. Typically, this is not a complication during the first months after surgery. Roeder

reported a median time to local recurrence of 20 months after initial treatment [79]. The presence of a positive margin after surgery, possibly seen in more than half of patients operated [80, 81], undoubtedly increases the risk of local recurrence [82], particularly in view of the recent advances in surgical techniques as extensively discussed above [29] with respect to the definitions of borderline resectable adenocarcinoma of the pancreas. There is not much information in the radiology literature so far detailing and describing local tumor recurrence patterns after pancreaticoduodenectomy, nor have the most common sites of local recurrence been adequately demonstrated and reported. One reason for lack of high quality follow-up imaging study may be that recurrence of pancreatic cancer was not treated, but in recent years radiochemotherapy and, in rare cases, surgery for local recurrence has been advocated [83]. A major problem in patients with pancreatic cancer is that extensive postoperative changes with scar tissue formation as well as lymph node enlargement are present after surgical therapy that may be mistaken for disease recurrence as already discussed above. One study was able to demonstrate, though, that a specific pattern of regrowth on regular follow-up MDCT might be identified after the Whipple operation for pancreatic adenocarcinoma. The mean follow-up interval was 3.9 $\pm$ 1.8 months, with a mean relapse-free interval of 12.9 $\pm$ 10.4 months. The predominant site of recurrence was local (65%), followed by lymph node (17%), liver metastasis (11%), and peritoneal carcinosis (7%). Local recurrence was identified at the superior mesenteric artery ($n = 28$), the hepatic artery ($n = 8$), in an area defined by the surrounding vessels: celiac trunk, portal vein, inferior vena cava ($n = 22$), and in a space limited by the mesenteric artery, portal vein and inferior vena cava ($n = 17$). Lymph node recurrence occurred in the mesenteric root and left lateral to the aorta [84]. The most important imaging finding was focal increase of hypoattenuating tissue strands alongside of the major vessels and within or near the previous resection margins in the mesentery. This corresponds with findings of careful and standardized microscopic exploration of the resection margins in resected specimen [80]. The superior mesenteric artery is the leading structure for recurrence [84]. As another result of this study, early detection of local tumor recurrence bears the potential of another surgical exploration with a secondary curative intent.

Conclusion

Early tumor detection, profound and precise differential diagnostic strategies, correct staging and adequate peri- and posttherapeutic imaging schedules must play a very important role in present and future treatment of pancreatic adenocarcinom. Early tumor detection is one of the key factors for a potential cure by surgical resection. Major advances in MDCT (multidetector computed tomography), including 2D and 3D reconstruction, are highly useful in improving staging and postsurgical care. For tumor detection, MDCT is applied in the dual phase technique using the different attenuation pattern of tumorous tissue versus normal pancreatic tissue, the former being specifically characterized by hypoattenuation both during the arterial and

venous phase acquisition. Such hypoattenuation is the imaging surrogate of the desmoplastic tissue character of pancreatic adenocarcinoma, which develops as a consequence of very specific biochemical and micromolecular behavior of pancreatic tumor cells. CT finds its limits when such hypoattenuation is lacking in small tumors. Then, MRI might be superior to MDCT. PET-CT is particularly helpful in differentiating between malignant and benign in complex clinical problems, such as discriminating between autoimmune pancreatitis and pancreatic adenocarcinoma, identifying the distant metastatic disease in the pretheraupeutic staging workup, and, furthermore, discriminating between benign fibrotic tissue and tumor recurrence in the follow-up after surgical resection. In the regular diagnostic workup, otherwise, it does not assume a major role.

Cross-References

- ▶ Arterial Resection in Pancreatic Cancer
- ▶ Clinical Decision-Making in Pancreatic Cancer
- ▶ EUS and Its Role in Pancreatic Cancer
- ▶ Interventional Radiology for Pancreatic Cancer
- ▶ MRI and MRCP for Diagnosis and Staging of Pancreatic Cancer
- ▶ Pathologic Classification and Biological Behavior of Pancreatic Neoplasia
- ▶ Role of Tumor-Stromal Interactions in Pancreatic Cancer Invasion and Metastases
- ▶ Staging and Postoperative Outcomes Using the International Study Group of Pancreatic Surgery (ISGPS) Classifications
- ▶ Stromal Inflammation in Pancreatic Cancer: Mechanisms and Translational Applications
- ▶ Surgical Resection for Pancreatic Cancer Using the International Study Group of Pancreatic Surgery (ISGPS) Classifications
- ▶ Venous Resection in Pancreatic Cancer Surgery

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MRI and MRCP for Diagnosis and Staging of Pancreatic Cancer

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Contents

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Abstract

Magnetic resonance imaging (MRI) has conventionally taken a secondary role to CT in the staging of pancreatic adenocarcinoma. It has been used for the evaluation of the pancreas in equivocal CT findings, in patients who are unable to have iodinated contrast media or to avoid using ionizing radiation.

However, MRI is particularly useful for the assessment of small pancreatic lesions, differentiating benign from malignant pancreatic lesions, and the assessment of cystic pancreatic masses, and has an invaluable role in the preoperative assessment prior to enucleation surgery. This chapter will cover the MRI sequences used for the diagnosis and staging of pancreatic neoplasms, the advantages and disadvantages of MRI, and will describe the mimics of pancreatic cancer, and other pancreatic neoplasms.

Keywords

MRI pancreatic carcinoma · Diffusion weighted imaging pancreatic carcinoma · MRI staging pancreatic carcinoma · MRI mimics pancreatic carcinoma · MRI technique for imaging pancreatic carcinoma

Introduction

Magnetic resonance imaging (MRI) of the pancreas has conventionally taken a secondary role in the diagnosis and staging of pancreatic malignancy.

The multidetector computed tomography (MDCT) pancreatic protocol has been extensively validated for the use of pancreatic staging for pancreatic carcinoma [1] and is the mainstay for the diagnosis and evaluation of surgical resectability. This is due to the superior spatial resolution of CT and the ability to multiplanar reformat the images for accurate tumor and vessel assessment.

However, due to its superior soft tissue contrast resolution, MRI has significant advantages over CT in the detection of small noncontour deforming pancreatic lesions, characterization and differentiation between benign and malignant lesions, and the detection and characterization of liver and peritoneal metastasis [2].

With increasing advancement in MRI technology, comparisons between CT and MRI have shown a similar ability in prediction of vessel and tumor involvement [3–5].

MRI Technique

The multipulse capability of MRI enables detection and characterization of pancreatic and liver lesions with a high degree of accuracy. Each individual sequence obtained provides tissue specific information of a lesion. The information gathered from all the sequences aids in the characterization of lesions where CT and conventional ultrasound cannot. This is particularly useful when endoscopic ultrasound with fine needle aspiration of the pancreas is not available or cannot be performed.

There are standard sequences used for the assessment of the pancreas, with occasional variations depending on the clinical question, and the age and capability of the MRI scanner available.

The standard sequences are outlined below.

T1 Weighted Gradient Recalled Echo (GRE)

The axial T1 weighted GRE sequence provides excellent delineation of the pancreatic contour demonstrating good anatomical detail. This is due to the inherent high signal of the pancreas on the T1 weighted sequences due to the presence of acinar cells with the pancreas, and the high content of paramagnetic ions such as manganese [6]. The pancreas is clearly outlined against the higher T weighted signal peripancreatic fat.

Fat suppressed T1 weighted sequences suppresses the macroscopic fat. The peripancreatic fat therefore becomes dark, thus increasing the conspicuity of the inherently high signal pancreas. This sequence is used for the post-contrast scans due to the high lesion contrast [7].

Most pancreatic abnormalities are low signal on the T1 weighted sequence including pancreatic lesions and pancreatitis, and therefore visible within the high signal pancreas. This enables the detection of small lesions (less than 2 cm) which can be beyond the resolution of CT. Difficulty may ensue when there is a pancreatic carcinoma within acute or chronic pancreatitis, as both pathologies return a low T1 weighted signal.

The T1 weighted sequences without fat suppression, where the surrounding peripancreatic fat is of higher signal to the pancreas is used to assess tumor infiltration into the fat and adjacent vessels.

T2 Weighted Sequences

On the T2 weighted fast or turbo spin echo (FSE/TSE) sequence, the normal pancreas is not as clearly defined as it has an intermediate signal intensity, only slightly higher than surrounding muscle. Solid pancreatic lesions are of low signal on this sequence making conspicuity with the pancreas difficult.

On fat suppressed images, there is little contrast differentiation between normal pancreas and surrounding peri-pancreatic fat.

However, fluid is bright on T2 weighted sequences. Thus, cystic lesions can be confidently identified within the pancreas, as can the outline of the pancreatic and biliary ductal system.

The presence of necrosis or cystic degeneration of a solid lesion is also more clearly identified on this sequence due to the internal fluid content.

On the T2 weighted sequences, the peri-pancreatic tissue is of a higher signal than the adjacent pancreas. This provides good delineation of the peri-pancreatic fat adjacent to pancreatic contour, enabling assessment of peri-pancreatic inflammation, peri-pancreatic tumor infiltration, and identification of lymph nodes.

Diffusion Weighted Sequences

The diffusion weighted sequence (DWI) is becoming an established sequence for the assessment of the pancreas due to its ability to provide information on the cellular density of tissue.

In normal tissue, water molecules diffuse freely in relation to molecular interactions and the cellular environment (Brownian motion). However, in the presence of pathology, this diffusion is restricted due to changes at the cellular level such as edema, fibrosis, or increased cellular density [8].

The diffusion weighted sequence is sensitive to molecular motion as it applies two "diffusion gradients" around the 180° refocusing pulse. Molecules that are restricted in their movement (due to cellular change) receive both gradient excitations and therefore receive no net change to their phase, and therefore return a high signal. Molecules that are unrestricted in their movement (normal tissue) do not receive both gradients excitations as they are motion, and therefore experience a phase loss and return a low signal.

The timing and application of these diffusion gradients determine the sensitivity to diffusion and is indicated by the use of "b-values," with increasing b-values indicating increasing sensitivity to diffusion.

A diffusion sequence will routinely begin with a b-factor of "b-0" to establish a baseline image and then with b-values of increasing value tailored to examine a particular tissue type. On images with a high "b" value, there is loss of anatomical detail of the solid organs, resulting in lesions with restricted diffusion appearing conspicuous.

The diffusion sequence in its natural form contains T2 contrast due to the repetition and echo time used in these sequences. The T2 contamination, termed "T2 shine through," can be misinterpreted as an indication of pathology if not fully understood and recognized. To correct this shine through, the calculation of apparent diffusion coefficients, or "ADC map" as it is better known, is required.

The ADC map is calculated using a logarithmic algorithm involving the b-0 and the second, or the multiple b-values acquired. Through the application of this algorithm, the effects of T2 shine through are removed, leaving a corrected image set. This ADC map is opposite to the initial uncorrected image set in signal properties: areas of restricted diffusion which have a high signal on the uncorrected

raw image set will have a low signal on the ADC map [9–11]. Quantification can be assessed on the ADC map using regions of interest.

As a result of the varied cellular densities of normal pancreas and pancreatic pathology, DWI can potentially be useful in the identification and characterization of pancreatic lesions.

Dynamic Contrast Scans

Dynamic contrast enhanced T1 weighted fat suppressed gradient recalled echo (GRE) sequences are performed following intravenous gadolinium contrast administration. The contrast enhanced sequences require patient cooperation with at least 4 to 5 breath-holds of at least 11 s in length.

An extracellular gadolinium agent is conventionally used for assessment of the pancreas. This behaves similarly to contrast agents in CT by diffusing rapidly from the intravascular space into the extracellular space. These are excreted by glomerular filtration via the kidneys.

Peak pancreatic parenchymal enhancement occurs at 35 s post-contrast resulting in intense homogenous pancreatic enhancement where lesion conspicuity is at its greatest. The pancreas then becomes isointense to the liver on the portal venous and delayed phases, with loss of contrast enhancement by 3 min. The pancreas is imaged 35 s (pancreatic parenchymal phase), 70 s (portal venous) and delayed phase scans, usually 1 and 3 min.

The contrast enhanced images also provides evaluation of the adjacent vessels for vascular staging.

Magnetic Resonance Cholangiopancreatography (MRCP)

MRCP is a fluid targeted sequence depicting the biliary and pancreatic ductal system. Two types of MRCP technique are utilized.

A thick slab single-shot turbo spin echo T2 sequence can be obtained in any plane with a single short breath hold. This provides an excellent overall view of the entire biliary and pancreatic ductal system.

The multisection thin slab single shot spin echo sequence requires breath-hold and therefore patient cooperation. This sequence provides more detailed view of the pancreatic duct providing thin slice sequential images.

To visualize the biliary tract and pancreatic duct without fluid from the surrounding stomach and duodenum obscuring the view, the patient is starved for at least 4–6 h and given a T2 negative oral contrast agent such as pineapple juice immediately before the scan. This effectively nulls the signal from the stomach and duodenum. On the MRCP sequences, the solid organ detail is not present, providing a clear view of the ductal system such as in ERCP.

The dorsal pancreatic duct is normally 2 to 3 mm in diameter, increasing caliber from the tail of the pancreas to head. Although there are several tiny side branches

arising from the pancreatic duct, these are not normally identified on MRCP unless pathologically dilated.

Cystic lesions and ductal abnormalities can clearly be identified on MRCP [12].

The presence of ductal narrowing may indicate the presence of a small pancreatic lesion and may be the only sign visible on imaging. Intraductal filling defects such as stones which are low signal compared to the high signal duct in patients with chronic pancreatitis are also clearly depicted as an alternative cause of ductal dilatation [13].

Secretin MRCP

Dynamic assessment of the pancreatic duct is possible with the administration of the enzyme secretin. This is an amino acid polypeptide hormone which is usually secreted by the duodenal mucosa in response to a meal when the intraluminal acidity increases. The synthetic version is administered by slow intravenous injection over 1 min in order to avoid side effects such as abdominal pain. The enzyme stimulates the production of pancreatic enzymes and increases the tone of the sphincter of Oddi, resulting in an increase in the caliber of the pancreatic duct. The increase in caliber can be seen by 1 min post-intravenous administration of secretin and reaches a maximum by 3–5 min, returning to normal by 5 min post-intravenous administration.

This sequence is used as an adjunct to conventional MRCP. The standard MRCP sequences are obtained followed by the dynamic images using coronal single shot turbo spin echo sequences every 30 s for 10 min postinjection. Although secretin MRCP is not used in diagnosis or staging of pancreatic adenocarcinoma, the transient increase in pancreatic duct diameter (usually by 1 mm or more) improves the depiction of the ductal anatomy and allows differentiation of a side branch IPMN from a mucinous tumor with a high degree of accuracy [14]. This will be discussed later on in the chapter.

This sequence can also be useful in the assessment of patency of the postoperative pancreaticoenteric ductal anastomosis.

Advantages and Disadvantages of MRI

MRI does not employ the use of ionizing radiation as in other imaging modalities, which allows investigation to be performed with no known biological harm to the patient. This is useful for pregnant patients and for patients who have multiple interval scans of the pancreas.

The main disadvantage of MRI is the length of the examination and the requirement for patients to take multiple breath-holds in order to obtain high-resolution diagnostic images.

The length of a typical MRI examination of the pancreas is around 30 min which can be a limiting factor for patients who are claustrophobic, in pain, or acutely unwell. Movement or breathing during the acquisition of the sequences can result in

significant degradation of imaging quality, thus reducing the diagnostic accuracy of the investigation.

Fast sequences can be utilized for patients who are unable to hold their breath, but to the detriment of diagnostic quality.

MRI Safety

MRI is particularly useful in imaging patients where administration of nonionic iodinated CT contrast media is contraindicated such as patients with a known allergy to CT contrast. The incidence of acute adverse severe reactions associated with MR gadolinium-based contrast agents varies between 0.17% and 2.4% [15]. This is significantly lower than the rate of adverse effects associated with nonionic iodinated contrast media [16, 17] and should be considered if the patient has an allergy to CT contrast. However, studies have shown that a previous reaction to CT contrast media does increase the risk for hypersensitivity reactions to gadolinium [18].

Risk factors for immediate hypersensitivity reactions to gadolinium contrast are noted in female patients, patients with underlying allergic diseases, multiple exposures, and those with a previous hypersensitivity to MR contrast media [19]. Patients with previous hypersensitivity to gadolinium are about eight times more likely to experience a reaction which can be of a greater severity than the initial contrast reaction [20].

Corticosteroid treatment has been used a premedication to reduce the incidence and severity of hypersensitivity reactions and is effective in preventing mild reactions [21]. However, patients who have had severe reactions are still at an increased risk despite premedication [22].

The administration of limited duration corticosteroids itself poses a risk particularly in patients with infection, diabetes, and hypertension.

Nephrogenic Systemic Fibrosis

In patients with renal failure, imaging with MRI and gadolinium contrast was previously considered to be a safe alternative to nonionic CT contrast media.

However, over the last decade, a condition called nephrogenic systemic fibrosis (NSF) has come to light. NSF is a fibrotic condition caused by the deposition of gadolinium within tissues of patients with end-stage renal failure [23].

The stability of the gadolinium chelate is directly linked with the development NSF. Plasma elimination of gadolinium from the body is approximately 2 h in patients with preserved renal function. However, in patients with renal failure, plasma elimination is lengthened. This increases the risk of displacement of the gadolinium ion from its chelating ligand and the formation of gadolinium-phosphate complexes which precipitate in tissues resulting in a fibrotic response [24, 25]. The exact parameter leading to lack of stability of the gadolinium chelate is not definitively known, with a lack of consensus in the literature [26].

Due to the accumulation of fibrosis in skin and visceral tissues, skin thickening, particularly involving the extremities, is noted with the development of joint contractures and loss of mobility [27]. Fibrosis involving the liver, lungs, muscle, and heart has also been recognized [28]. In some patients, this disease can be aggressive, leading to severe disability or death.

NSF has been seen in patients with chronic renal failure with an eGFR less than 30 mL/min, resulting in an incidence of NSF in 3–5% in these patients [29, 30]. Patients with hyperphosphatemia, acidosis, or pro-inflammatory states are also at increased risk [31].

Recommendations have been published by the European Society of Uroradiology (ESUR), American College of Radiology (ACR), and Food and Drug Administration (FDA) on the use of gadolinium contrast. Some gadolinium agents are contraindicated in patients with acute and chronic renal failure (CKD 4–5) as they have the highest association with NSF: gadopentate (Magnevist), gadodiamide (Ominiscan), and gadoversetamide (optiMARK). The other gadolinium agents are recommended to be used in caution in patient with low eGFR (<30 mL/min), and multiple administration of gadolinium to be avoided within a 7-day period.

Other recommendations vary between ESUR and ACR on the use of other gadolinium agents, and the timing of dialysis post-gadolinium administration [32].

Current guidelines for patients undergoing MRI with contrast include assessment of the eGFR in patients over 60 years, a history of renal disease, hypertension, or diabetes.

Referral to these guidelines online is suggested for the most up-to-date information in the relevant country of residence.

Diagnosis and Staging of Pancreatic Adenocarcinoma

Tumor Diagnosis

On T1 weighted sequences, pancreatic adenocarcinoma is demonstrated as an ill-defined hypointense mass within the high T1 signal pancreatic parenchyma. Thus, small lesions beyond the resolution of CT or iso-attenuating lesions on CT are better defined on this sequence [33]. This is potentially useful when EUS is not available or cannot be performed.

Tumor infiltration into the peri-pancreatic tissue is depicted as a low signal mass among the high signal fat on the nonfat saturated T1 weighted sequence. This is usually depicted as nodular infiltration into the fat or along the peri-pancreatic vessels, or vascular encasement.

On the T2 weighted sequences, pancreatic adenocarcinoma is isointense to mildly hyperintense compared to background pancreas due to its fibrotic nature. This makes identification of the lesion within the pancreatic parenchyma difficult. The presence of necrosis or cystic degeneration may help visualization as this will return a high signal compared to background pancreas. Assessment of the dilated pancreatic duct and its transition point is an important secondary sign of the presence of a mass lesion, and is well visualized on this sequence and on MRCP.

Administration of intravenous gadolinium contrast increases the conspicuity of tumors and improves the detection rate of small tumors (less than 2 cm) [34, 35].

After administration of intravenous gadolinium contrast, pancreatic adenocarcinoma demonstrates decreased enhancement compared to the pancreas on the pancreatic parenchymal phase image (35 s), with mild progressive enhancement into the delayed sequences. This is due to the desmoplastic nature of the lesion [36]. However, the tumor remains lower signal than the surrounding enhancing pancreas. This is differentiated from inflammatory lesions which demonstrate increased enhancement compared to the pancreas on the delayed contrast enhanced scans.

Diffusion sequences have been shown to be useful in the identification of the pancreatic adenocarcinoma from background pancreas by visual assessment on the DW images and by quantification on the ADC map [37–40]. Pancreatic adenocarcinoma is bright on the high "b" value DW images compared to the background pancreas and returns a lower ADC value on quantitative analysis.

Small pancreatic tumors have been shown to demonstrate restricted diffusion, as shown in cases of neuroendocrine tumors [41].

In patients with chronic pancreatitis, identification of adenocarcinoma may not be reliable. The inherent high signal of the pancreas on the T1 weighted sequence is lost in both pathologies, making differentiation on this sequence difficult. Chronic pancreatitis may also appear hyperintense on the high b value DW images making visual assessment on this sequence misleading [42]. However, ADC values have been shown to differ with adenocarcinoma returning a lower ADC value than chronic pancreatitis, and can be useful if there is a high index of suspicion of adenocarcinoma within chronic pancreatitis.

Although diffusion weighted imaging may be useful in differentiating benign from malignant mass lesions [37, 38], to date, there are only a few studies looking at characterization of different solid pancreatic lesions using DWI. Studies have shown there is a wide overlap in ADC quantification in differing solid pancreatic lesions making accurate characterization difficult [40, 43]. Studies have also looked DWI of adenocarcinoma with different histopathological grades, but the findings are still unclear if DWI can be helpful here [8].

Occasionally the primary malignancy can be difficult to appreciate on imaging. The secondary signs of pancreatic adenocarcinoma include pancreatic duct dilatation, atrophy of the pancreas distal to the tumor, and dilated collateral vessels due to venous invasion of the tumor. These signs can be the only indication of the presence of a mass (Fig. 1).

Vascular Resectability

The extent of vascular involvement by pancreatic adenocarcinoma is best depicted on post-contrast multidetector CT imaging with 3-D reformats. Gadolinium-enhanced MRI is inferior to multidetector CT in terms of spatial resolution and 1.5 T MRI does not provide isotropic imaging in order to obtain 3-D reformatting.

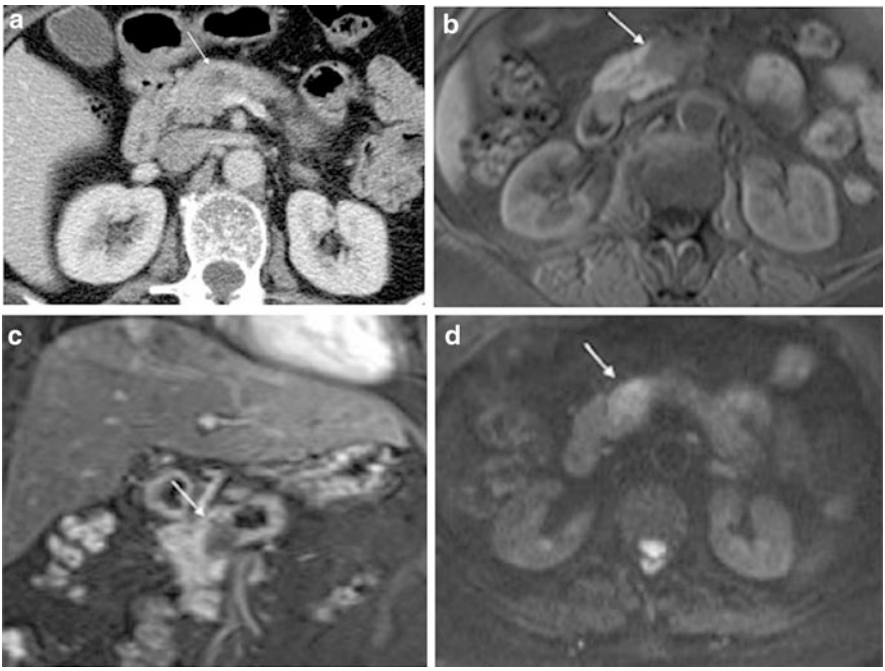

Fig. 1 CT and MR findings of a pancreatic adenocarcinoma in the neck of pancreas. (**a**) Axial contrast enhanced CT demonstrating a nonspecific hypo/ iso-dense swelling of the head of pancreas with no defined mass (*white arrow*). (**b**) Axial T1w noncontrast image demonstrating a hypointense mass (*white arrow*) within the pancreas. (**c**) Post-contrast coronal T1w fat saturated image demonstrating a hypoenhancing mass with a normally enhancing pancreatic head. (**d**) Diffusion weighted images at a high "b" value demonstrating restricted diffusion (*white arrow*) (Images courtesy of Dr. R Albazaz, Leeds Teaching Hospital NHS trust)

However, with the advent of 3.0 T MRI scanners, 3-D gradient echo images have become available enabling reconstructions of 1–1.5 mm slice thickness in order to obtain accurate vascular assessment. Here, MRI with MR angiography has shown to have similar sensitivities of determining resectability compared to multidetector CT (approximately 90%) [44]. Assessment for vascular staging is the same for CT staging and is described in the chapter ▶ "Pancreatic Adenocarcinoma: CT and PET/CT" (Fig. 2).

Assessment for Enucleation of Pancreatic Lesions

Enucleation surgery has been performed for small cystic tumors, neuroendocrine lesions, and IPMN.

MRI is particularly useful in the surgical assessment of the lesions. The combination of the T1 and T2 weighted images allows accurate assessment of the distance

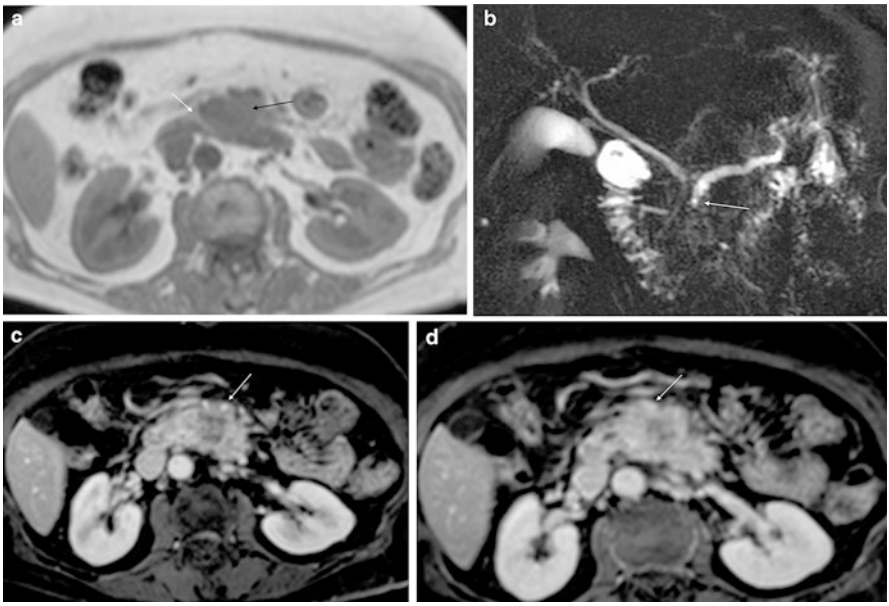

Fig. 2 MR images demonstrating an uncinate process mass with vascular compromise. (**a**) Axial T1 in-phase scan demonstrating the low signal uncinate process mass (*black arrow*) with rim of normal high T1 signal pancreas (*white arrow*). (**b**) MRCP sequence demonstrating a dilated pancreatic duct with sharp cutoff at the uncinated process of pancreas (*white arrow*). (**c**) Axial T1 fat saturated post-contrast arterial phase scan demonstrating the hypoenhancing uncinate process tumor with encasement of the superior mesenteric artery. (**d**) Axial T1 fat saturated post-contrast portal venous phase scan demonstrating the hypoenhancing uncinate process tumor with involvement of the posterior wall of the superior mesenteric vein (*white arrow*)

of the pancreatic lesion from the pancreatic duct to avoid involvement of the duct during surgery.

Nodal Disease

Accurate nodal staging has been shown not to be reliable on cross-sectional imaging. Where size criteria were historically used to differentiate benign from malignant nodes, this has shown not to be accurate [45] with presence of micro-metastases occurring in normal appearing lymph nodes. Nodes are more difficult to see on MRI sequences than CT, but the presence of necrosis within a node does significantly increase the suspicion of malignant infiltration.

Liver Metastases

MRI is able to detect liver lesions with a high degree of sensitivity (81–92%) compared to multidetector CT (70–87%) [46]. The addition of diffusion weighted

sequences has led to the ability to detect very tiny liver lesions not seen on other modalities or on other MR sequences [47, 48].

Characterization and detection of liver lesions is significantly increased with the use of hepatocyte specific contrast agents (gadoxetate disodium, *Primovist*, *Bayer*, *Germany* or gadobenate dimeglumine, *MultiHance*, *Bracco*, *Princeton*, *NJ*). This type of contrast agent is taken up by the hepatocytes and is excreted via hepatobiliary system.

The enhancement of liver on the dynamic contrast arterial, portal venous, 1 and 5 min delayed phase scans is similar to the other extracellular gadolinium contrast agents. Specific liver uptake of the contrast by hepatocytes results in optimal contrast enhancement of the liver on the delayed phase scans (20–40 min for gadoxetate disodium, and 60 min for gadobenate dimeglumine). Smaller liver lesions are clearly delineated against the uniformly enhancing background pancreas. Due to the excretion of contrast by the hepatobiliary system, the biliary tract is also well visualized on the delayed scans.

Liver metastasis secondary to pancreatic adenocarcinoma tends to be hypovascular. The lesions are hypointense on T1 weighted sequences, iso- to moderately hyperintense on T2 weighted sequences, and can have a target appearance. They demonstrate irregular rim enhancement post-contrast (Fig. 3).

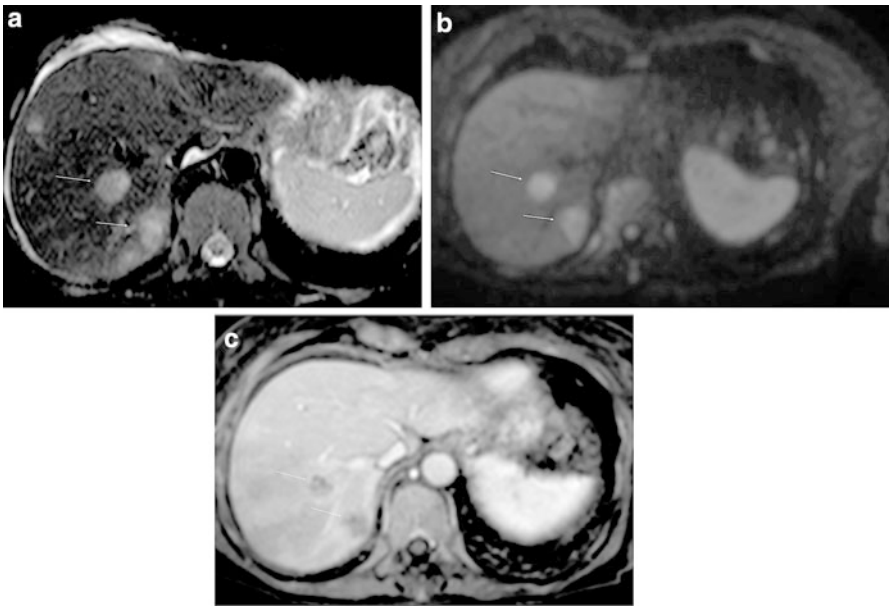

Fig. 3 Selected images demonstrating liver metastases secondary to pancreatic adenocarcinoma. (**a**) Axial T2 weighted images of the liver demonstrating several liver metastases with a target-like appearance. Two of the lesions have been arrowed with a *white arrow*. There is also ascites in the upper abdomen. (**b**) DWI images demonstrating restricted diffusion of these visualized two lesions on this slice (*white arrow*). (**c**) Axial T1 weighted fat saturated post-contrast scans demonstrating irregular rim enhancement of these metastases (*white arrow*)

Wedge-shaped perilesional transient enhancement on the arterial phase is seen typically with pancreatic adenocarcinoma metastases, and not seen in neuroendocrine liver metastases. Pancreatic adenocarcinoma liver metastases in the periphery of the liver tend to be hypervascular and maybe only seen transiently on the arterial phase scan [49].

Tumor Assessment Post-neo-Adjuvant Chemotherapy

Patients with borderline resectable disease who have been treated with chemotherapy to downstage the tumor have repeat imaging prior to surgical consideration. In both CT and MRI imaging, the posttreatment fibrosis results in over-staging of local disease with both CT and MRI demonstrating a reduced sensitivity and specificity in predicting vascular involvement and resectability post-chemotherapy [50].

Mimics of Pancreatic Adenocarcinoma

Fatty Changes

Fatty replacement within the pancreas is usually diffuse, but not so rarely, can be focal and typically present in the anterior aspect of the pancreatic head. This can mimic a mass on CT or ultrasound. Due to the availability of fat and nonfat suppressed sequences, MRI is of choice for definitive diagnosis.

On the T1 weighted sequence, the fatty lesion is typically iso- or hyperintense to the pancreas. On the T1 fat saturated sequences, the area of fat will suppress appearing low signal compared to the remainder of the pancreas [51]. Post-contrast, there is homogenous enhancement of the pancreas, thus differentiating focal fatty change from adenocarcinoma.

Mass-Forming Pancreatitis

Focal pancreatitis and pancreatic adenocarcinoma can be difficult to differentiate on imaging and may lead to unnecessary surgical resection. In the absence of tissue confirmation by EUS FNA, MRI can be useful in differentiating the two pathologies.

Focal pancreatitis is usually more defined than pancreatic adenocarcinoma but also returns a low signal on T1 weighted sequence. As with adenocarcinoma, pancreatitis demonstrates reduced enhancement compared to the background pancreas, but demonstrates progressive enhancement on the delayed contrast images, more so than adenocarcinoma, and can enhance to a greater extent than the normal pancreatic tissue. Subtle findings also include preservation of pancreatic architecture if the inflammation is not marked, whereas this architecture is destroyed in adenocarcinoma.

However, it can be impossible to differentiate the two pathologies on imaging, and follow-up imaging in about 4–6 weeks is advised if the clinical features favor pancreatitis.

Mass-forming autoimmune pancreatitis (AIP) is another mimic of adenocarcinoma on both imaging and histology. Homogenous enhancement of mass-forming AIP on the arterial and portal venous phase sequences differentiate this from pancreatic adenocarcinoma, as well as the preserved architecture of the pancreas. The duct penetration sign, where the main pancreatic duct penetrates the mass is a specific finding in an inflammatory pancreatic mass lesion. This appearance is different to pancreatic adenocarcinoma where there is an abrupt cutoff of the pancreatic duct at the site of the mass (Fig. 4).

Lower ADC values in mass-forming AIP have also been shown to be useful in differentiating the two pathologies, but this finding has not always been consistent in the literature with substantial overlap in the ADC values [52]. Other features of AIP are the halo sign, with a thin rim of fluid around the pancreas and evidence of autoimmune disease in other organs.

Other Solid Pancreatic Tumors

Solid Pseudopapillary Tumor

Solid pseudopapillary tumor of the pancreas represents 1–2% of pancreatic tumors. These are of low-grade malignant potential and predominantly occur in younger women. They are usually large (mean 9 cm), located within the tail and are well demarcated with a thick solid capsule which enhances post-contrast. On the dynamic contrast enhanced scans, there is variable enhancement ranging from a hypervascular mass with washout to slow enhancement on the arterial phase with progressive enhancement to into the delayed phase [53]. The mass tends to displace surrounding structures rather than invading them. Due to hemorrhage, the lesion can exhibit solid and cystic components, and as a consequence demonstrate high signal on the T1w sequence and appear cystic on the T2w sequence, differentiating this from pancreatic adenocarcinoma [54].

Pancreatic Neuroendocrine Tumors (NET)

Functioning NET are identified primarily from symptoms due to the secretion of hormones rather than identification of a mass on imaging. These lesions tend to be small at diagnosis (less than 3 cm) and can be elusive on cross-sectional imaging.

These lesions are well defined, low signal on the T1 weighted sequences, but demonstrate higher signal on the T2 weighted sequences compared to the

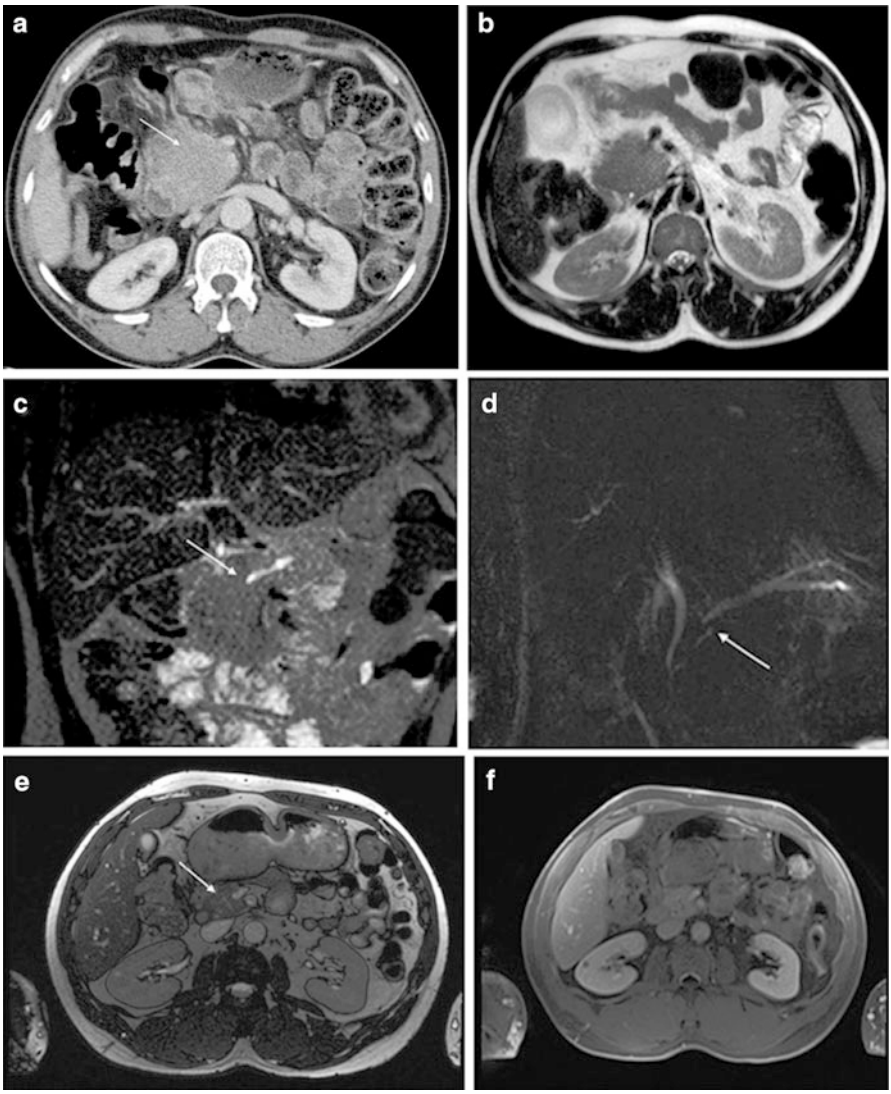

Fig. 4 Selective images of a mass forming AIP pre and posttreatment. (**a**) Axial contrast enhanced CT scan demonstrating a slightly hypodense expanded pancreatic head (*white arrow*) in keeping with a mass. (**b**) Axial T2 weighted sequence of the head of pancreas which is slightly higher signal than muscle. (**c**) Coronal T2 weighted sequence demonstrating the high signal pancreatic duct penetrating the mass (*white arrow*). (**d**) MRCP sequence demonstrating the dilated pancreatic duct with a tapering and penetrating into the pancreatic head (*white arrow*). (**e**) Axial T2 weighted scan of the pancreatic head which appears normal in size with a normal pancreatic duct (*white arrow*), 4 weeks post-steroid treatment. (**f**) Axial portal venous phase scan of a normally enhancing pancreatic head (Images courtesy of Dr. R Albazaz, Leeds Teaching Hospital NHS trust)

background pancreas and can appear cystic with a thickened wall. They are hypervascular demonstrating intense arterial enhancement post-contrast. They can also demonstrate ring enhancement. Malignant endocrine neoplasms tend to demonstrate restricted diffusion, but the ADC values do to vary due to tumor differentiation, hemorrhage, and necrosis [55] (Fig. 5).

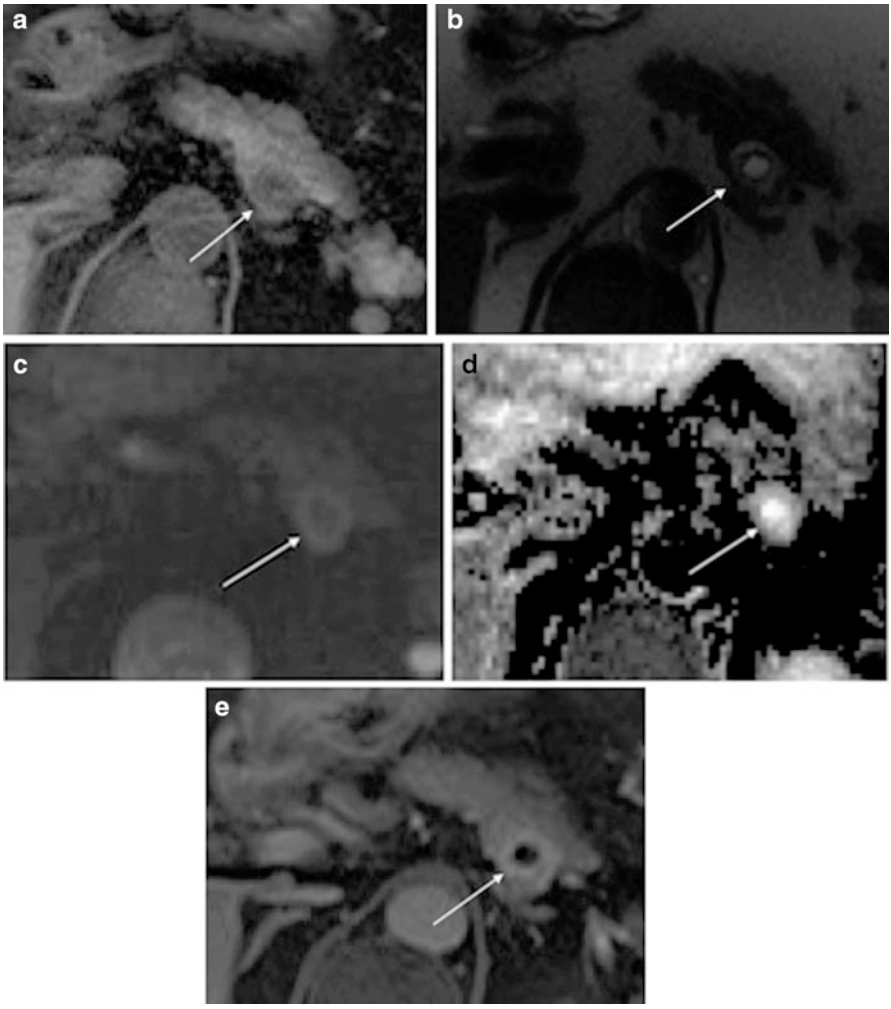

Fig. 5 Selected images of a NET of the posterior body of pancreas with cystic degeneration. (**a**) Axial T1 fat saturated images demonstrating the low signal lesion within the high signal pancreas (*white arrow*). (**b**) Axial T2 image demonstrates the lesion returning a high signal. (**c**) DWI images with restricted diffusion of the rim of the lesion (*white arrow*). (**d**) ADC map with low signal rim in keeping with restricted diffusion (*white arrow*). (**e**) Axial post-contrast portal venous image demonstrating rim enhancement of the NET (*white arrow*) (Images courtesy of Dr. R Albazaz, Leeds Teaching Hospital NHS trust)

Nonfunctional endocrine tumors tend to be much larger in size at presentation due to the lack of hormone secretion and symptoms. These lesions exhibit calcification, cystic/necrotic degeneration, and vascular invasion. The vascular invasion tends to be fingerlike intravascular solid tumor thrombus within the affected vessels, a feature not usually seen in patients with adenocarcinoma. Enhancement is varied due to the necrosis and calcification, but the solid areas are typically hypervascular [56, 57].

Lymphoma

Primary pancreatic lymphoma is rare and seen usually in immunocompromised patients. This is commonly the B cell type of non-Hodgkin's lymphoma and can either be a focal well-circumscribed lesion or a diffuse infiltration of the pancreas.

The focal type of lymphoma typically localizes at the pancreatic head with no significant dilatation of the main pancreatic duct. There can be encasement of the vessels but vascular distortion is not seen.

The diffuse form of pancreatic lymphoma can mimic acute pancreatitis.

The imaging characteristics are nonspecific, demonstrating low signal on T1 and intermediate signal on T2 weighted images, and demonstrating faint contrast enhancement [58].

Metastases to the Pancreas

Metastasis to the pancreas is relatively rare. Renal cell carcinoma metastases have a predilection for the pancreas (30%). Other malignancies include bronchogenic carcinoma (23%), breast, and colon. Renal cell carcinoma metastases are hypervascular on the arterial phase. Otherwise, metastases have variable heterogeneous enhancement and can be difficult to differentiate from adenocarcinoma [59]. However, the patient will have a history of current or previous malignancy, and the lesions may be multiple, which is not typically seen in adenocarcinoma.

Cystic Lesions of the Pancreas

The majority of cystic lesions within the pancreas are discovered incidentally on imaging, either ultrasound, CT or MRI. The incidence of these cysts is increasing, and may be in part due to the availability of high-end ultrasound, CT, and MRI scanners, and a general increase in diagnostic imaging of the population. Only rarely, are these pancreatic cystic abnormalities malignant mucinous lesions.

Serous Cystic Lesions

Serous cystadenoma of the pancreas is considered to be a benign entity, seen in older female patients with only very rare cases of malignant degeneration.

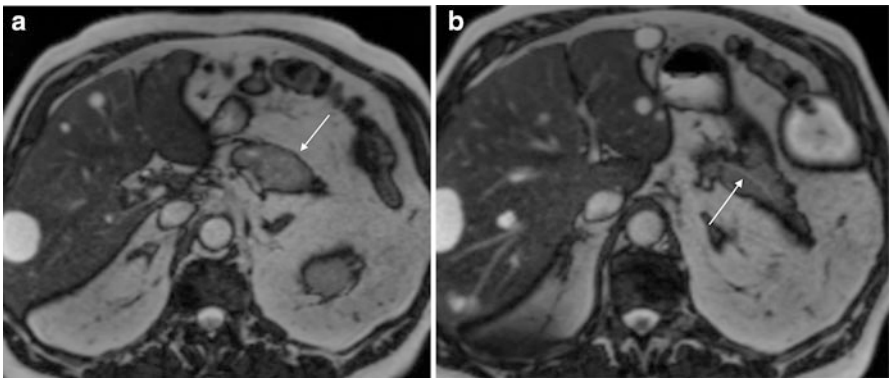

Fig. 6 Selected images demonstrating a serous cystic lesion in the tail of pancreas. (**a**) Axial T2 weighted image demonstrating a mass with several small cysts and a central low signal scar (*white arrow*). (**b**) Axial T2 weighted image demonstrating a normal caliber distal pancreatic duct (*white arrow*) and no pancreatic atrophy

These lesions typically contain multiple tiny cysts (less than 2 cm) with a central stellate calcified scar giving a honeycomb appearance. On CT, these can look solid and can mimic pancreatic adenocarcinoma. On MRI, the multiple cysts are clearly delineated on the T2w sequences where fluid is bright, with the low signal delayed enhancing central scar enabling a confident diagnosis. There is no dilatation of the pancreatic duct and no pancreatic atrophy (Fig. 6).

Mucinous Tumors of the Pancreas

Mucinous cystic neoplasms occur more often in women, seen within the body or tail of the pancreas, and have a higher malignant potential. These lesions have a range of histology. The most recent WHO update classifies lesions from benign mucinous lesions with low to intermediate grade dysplasia (previously termed cystadenoma), to mucinous lesions with high grade dysplasia (previously termed cystadenocarcinoma), and mucinous tumors with associated invasive carcinoma.

Mucinous cysts are larger than other neoplastic cysts, lobulated and exophytic and are typically unilocular with a few septations. The lesions have thick enhancing walls with septations, calcifications, and occasionally solid papillary excretions. Again, these are usually high signal on the T2 sequences but given their mucin component, can demonstrate variability in signal characteristics, including high signal on T1 weighted images.

These are differentiated from side branch intraductal papillary mucinous neoplasms (IPMN) by lack of connection to the main pancreatic duct on the MRCP sequences [60].

Increased risk factors for adenocarcinoma or high-grade dysplasia in mucinous cystic neoplasms are the male sex, pancreatic head and neck location, larger lesions, solid components or mural nodules, and pancreatic duct dilatation [61].

Intraductal Papillary Mucinous Neoplasms (IPMN)

IPMN arise from the main pancreatic duct or the side branches. Three types of IPMN are recognized: the side-branch IPMN, main branch IPMN, and mixed type IPMN.

MRCP imaging is the most useful noninvasive imaging modality to assess for IPMN. The pancreatic duct and side branches are well delineated on the T2 weighted sequences and MRCP sequences.

Side branch IPMN are most commonly identified in the uncinate process of the pancreas and appear septated or lobulated. However, they can be found elsewhere within the pancreas and can appear as unilocular cystic foci. Although more commonly solitary, they can be multifocal in 40% of cases.

The presence of a side-branch IPMN can be reliably depicted where communication between a cystic lesion and the main pancreatic duct is shown. However, this may not be reliably identified on imaging.

Studies have shown a low risk of malignancy if there are no solid components, no dilatation of the main pancreatic duct and the cysts measure less than 3 cm [62, 63].

Worrisome features of a cystic lesion in the pancreas include a cyst of greater than 3 cm, thickened enhancing cyst wall, abrupt change in the caliber of the main pancreatic duct with distal pancreatic atrophy, nonenhanced mural nodules, and lymphadenopathy. Cysts with high risk stigmata are lesions with an enhancing solid component and a main pancreatic duct greater than 10 mm [64] (Fig. 7).

Main branch IPMN carries a higher risk of malignancy of between 23% and 57% [65] and management is often surgical. Features include dilatation of the main pancreatic duct of more than 5 mm, either diffuse or segmental dilatation in the absence of an obstructive lesion. The side branches can be dilated, and small mural nodules can be identified. The pancreatic parenchyma becomes atrophied, particularly with increasing ductal dilatation.

The main branch IPMN type is clearly depicted on the MRCP and T2 weighted sequences and accurate measurements can be performed to demonstrate the extent and caliber of dilatation and stricturing of the pancreatic duct.

The excellent soft tissue contrast between high signal fluid and low signal soft tissue on the T2 weighted sequences of MRCP enables accurate detection of solid papillary projections and mass formation within an IPMN undergoing malignant transformation.

The presence of a solid mass, dilatation of the main pancreatic duct to over 10 mm diameter, diffuse or multifocal involvement, and calcified intraluminal content are specific signs of malignancy [66].

MRI is the preferred imaging modality for the follow-up and management of IPMN due to the superior delineation of these lesions on the T2 weighted and post-contrast sequences. The lack of ionizing radiation allows for repeated interval imaging without risk of radiation burden to the patient [67, 68]. The management of cystic neoplasms is discussed in chapter ► “Management of Cystic Neoplasms of the Pancreas Including IPMNs”.

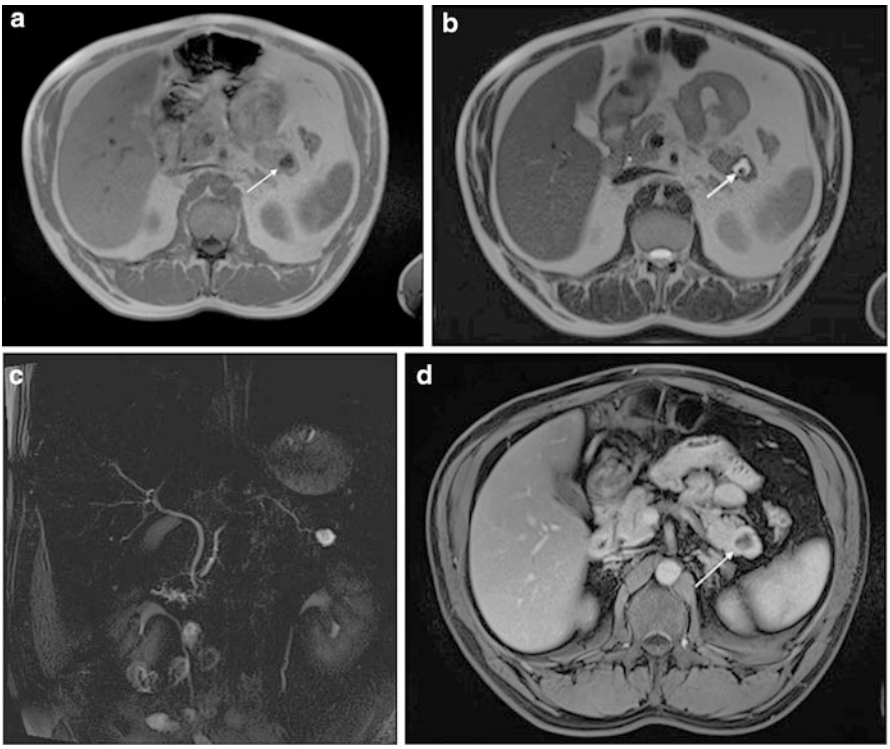

Fig. 7 Selected images of a pancreatic cyst in the tail of pancreas with a mural nodule. (**a**) Axial T1 sequence demonstrating a low signal lesion in the tail of pancreas (*white arrow*). (**b**) Axial T2 sequence demonstrating the cyst with a mural nodule (*white arrow*). (**c**) MRCP sequences demonstrating the cyst with internal mural nodule, and good overview of the pancreatic and biliary ductal system. (**d**) Axial contrast enhanced scan in portal venous phase demonstrating thick rim enhancement and mild enhancement of the mural nodule

Conclusion

With recent advances in the technology of magnetic resonance imaging, MRI is being increasingly utilized in the imaging of pancreatic lesions. It is a particularly useful problem-solving tool in the evaluation of pancreatic cysts, identification of small pancreatic lesions beyond the resolution of CT, and has increasingly potential use in differentiating benign from malignant pancreatic lesions. The ability to clearly visualize the pancreatic duct and define this from a pancreatic lesion makes MRI invaluable in the preoperative assessment prior to enucleation surgery.

For staging, with the advent of 3 T MRI, the ability of vascular staging is becoming comparable to CT. MRI with diffusion weighted imaging and gadolinium contrast has been shown to be far superior to CT in the detection of liver metastases,

and its use prior to consideration of pancreatic surgery may have a significant impact on patient outcome.

However, MRI is not without its risk, particularly for contrast enhanced scans. Patients with known relevant risk factors must be assessed prior to consideration of contrast enhanced MRI and the consequences may be severe.

Cross-References

► Management of Cystic Neoplasms of the Pancreas Including IPMNs
► Pancreatic Adenocarcinoma: CT and PET/CT

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EUS and Its Role in Pancreatic Cancer

Tobias Grote and Thomas Mathias Gress

Contents

Abstract

Endosocopic ultrasound (EUS) has become an indispensable tool in pancreatic diseases especially in cancer. This article provides an overview about basic principles as well as current developments in the field. It reviews recent literature regarding the use of EUS in pancreatic cancer. The key focus is on EUS-guided tissue sampling by EUS-fine-needle aspiration (EUS-FNA). Further main aspects include cystic pancreatic neoplasias, screening of individuals at risk for familial pancreatic cancer, and EUS-guided therapy.

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Introduction

Endoscopic ultrasound (EUS) was developed in the late 1970s and allows to visualize the complete pancreas without interfering signals from the overlying gas, which is a main obstacle in transabdominal ultrasound. In the early 1990s EUS-guided fine-needle aspiration (EUS-FNA) added an important tool in diagnosing pancreatic cancer. In recent years EUS-FNA has also gained significance as a standard, whenever a tissue diagnosis is required, e.g., before starting palliative chemotherapy, but also before initiating neoadjuvant treatment in resectable disease. Recommendations on the role of endoscopy including EUS and EUS-FNA in the evaluation and management of patients with solid pancreatic neoplasia have recently been summarized by the ASGE Standards of Practice Committee [1].

EUS Equipment

EUS imaging can be performed with radial (360°) or linear echoendoscopes. Some aspects of the different instruments have been recently reviewed [2]. Nowadays the EUS probes are coupled to electronic ultrasound processors for the generation of electronic EUS-images, endowed with special aspects as Doppler, contrast-enhanced endoscopic ultrasound, harmonic imaging, and elastography. Frequency usually varies between 5 and 10 MHz. Small miniprobes have been developed that are introduced through the working channel of conventional endoscopes and can be advanced into the biliary or pancreatic duct. These probes use high-frequency ultrasounds (12–30 MHz); however, they are not widely used. Radial and linear echoendoscopes are both used for the evaluation of pancreatobiliary diseases and perform equally well. Some experts only use linear probes as they allow performing fine-needle aspiration in the same procedure. Recently, in a randomized tandem study, it has been suggested that linear array EUS may have advantages in detection of pancreatic lesions in high-risk individuals [3]. However, extensive personal experience and training with one or the other EUS probe certainly remains among the most important criteria for the quality of EUS results.

Visualization and Staging of Pancreatic Cancer with EUS

Usually EUS is used for the evaluation of a pancreatic tumor that has been detected or is suspected in another imaging modality such as abdominal ultrasound or CT. When it is used for pancreatic cancer screening, e.g., in research programs for

individuals at risk for familial pancreatic cancer, EUS may be the first imaging modality detecting a tumor. Pancreatic cancer can be identified by EUS as homogenous or inhomogeneous echo-poor area, sometimes with echo-rich spots or cystic components [4]. More advanced tumor stages display an even less homogeneous tissue pattern and infiltrate neighboring organs and large peripancreatic vessels. While most surgeons will rely on modern CT imaging studies as standard to assess tumor resectability, criteria have been developed to evaluate vascular involvement using EUS. These criteria comprise the lack of a hyperechoic interface between the vessel wall and the tumor, the detection of tumor material in the vessels, the visualization of collaterals due to arterial or venous thrombosis, and indirect criteria such as tumor size or the proximity to major vessels. However, the significance of these criteria remains under debate [5].

Compared with CT and MRI, EUS is the more operator-dependent modality though in the hands of an expert EUS is the most sensitive test to detect pancreatic mass lesions that are less than 2 cm in size or equivocal in other imaging modalities [6]. A systematic review of nine studies [7] reached the conclusion that EUS is more sensitive than CT for the detection of pancreatic cancer (91–100% vs. 53–91%), whereas the two modalities deliver similar results for loco-regional tumor staging. EUS is usually performed prior to ERCP and stent insertion to avoid interfering signals of the biliary stent on the accuracy of EUS staging. However, the effects of biliary stents on staging accuracy appear to be negligible [8].

EUS-Guided Tissue Sampling

A new hallmark in endoscopic ultrasound in the beginning of the 1990s was the introduction of EUS-guided tissue sampling using steel needles [9, 10]. EUS thus allows tissue acquisition for pathology diagnosis, though in resectable tumors, sampling may not be necessary before surgery [11] and is, e.g., not routinely recommended in the German S3-guidelines for pancreatic cancer [12]. However, in some situations, a nonoperative pathology diagnosis in patients with otherwise resectable lesions may be essential. For example, endoscopic tissue diagnosis is helpful for the diagnosis of conditions that may mimic neoplasms or tumors such as autoimmune pancreatitis [13] and cystic lesions [14] or to allow patient enrollment into a neoadjuvant chemotherapy protocol [15]. For EUS-guided tissue sampling, thin steel needles are introduced through the working channel of linear echoendoscopes. In order to protect the instrumentation channel, the needles are covered by a plastic and metal-reinforced sheath, which extends out of the working channel. Once a stable position is reached and the target is in focus, the needle is advanced into the lesion passing through the gastrointestinal wall. The stylet is pulled back before the tip of the needle is pushed into the lesion. A negative pressure is applied using a syringe at the end of the needle. Now the needle is moved forward and backward several times within the lesion to obtain sufficient material for cytological and histological examination [4]. EUS-guided tissue sampling can be performed by FNA (EUS-guided FNA [EUS-FNA]) or by EUS-guided fine-needle

core biopsy (EUS-FNB). EUS-FNA has a sensitivity and specificity of up to 95% and 100%, respectively [16, 17], and is the preferred and a cost-effective method for making a definitive cytology diagnosis of a pancreatic mass [18]. Reasonable sensitivities for cytological analyses is usually obtained by performing five–six needle passes [19], though even two needle passes may already yield sufficient material for cytological and histological analyses [20]. Immediate evaluation and feedback from an on-site cytopathologist during sampling increases diagnostic yield by 10–15% [21, 22], however, due to logistic and infrastructural limitations is not widely available.

Several recent trials compared the diagnostic yield of EUS-FNA versus EUS-FNB [23, 24]. Though results are inconclusive, there seems to be a trend toward EUS-FNB, as this approach preserves the tissue architecture and may achieve a higher yield. A meta-analysis, though, could not demonstrate a significant benefit for core needles regarding sample adequacy and diagnostic accuracy [25]. EUS-FNA with cytopathology usually is adequate for a diagnosis of adenocarcinoma and neuroendocrine tumors (NETs), but it may not provide sufficient material to establish diagnoses such as lymphoma, well-differentiated carcinoma, or autoimmune pancreatitis [26]. Overall, several studies indicate that EUS-FNB is not superior to EUS-FNA in the work-up of a pancreatic mass, but should be considered if EUS-FNA is nondiagnostic and a histological diagnosis is needed [27–29]. FNB may be more technically challenging in particular for sampling of pancreatic head masses since the FNB needle has a higher stiffness which is less compatible with the angulation of the endoscope required for biopsy from this location. More flexible needles have been developed recently that may allow to obtain core tissue biopsies from the pancreatic head when required to establish the histological diagnosis [30].

Potential adverse events from EUS-guided sampling of pancreatic masses include a 0.5–2% risk of pancreatitis or bleeding [16, 17, 31]. In a prospective study of 3,207 diagnostic EUS (8% pancreatobiliary tumor staging) and 224 EUS-guided FNA (48% solid pancreatic masses), a morbidity of 0.1% ($n = 3$) and 2.2% ($n = 5$), respectively, with no mortality was reported [32]. Although pancreatitis is a dreaded complication of pancreatic EUS-FNA, the incidence was low in a large multicentric survey of 4,909 EUS-guided FNAs of solid pancreatic masses, with only 14 cases of acute pancreatitis reported (0.29%) [33]. Tumor seeding with EUS-FNA has been reported, but the risk appears to be as small as 0.003–0.009% [34], and reports are currently limited to isolated cases [35, 36]. Since in addition for pancreatic head masses the potential site of seeding is included in the resection, the small risk of seeding appears to be irrelevant. Indeed, preoperative EUS-FNA has not been reported to be associated with adverse perioperative or long-term outcomes in patients undergoing resections for solid neoplasms of the pancreas [37] (Figs. 1 and 2).

Novel Developments in EUS-Guided Tissue Sampling

EUS-FNA for cytological approach is deemed time consuming and often unable to provide suitable specimens for modern molecular analyses. A recent prospective study compared the cytological analysis of 130 specimens obtained by EUS-FNA

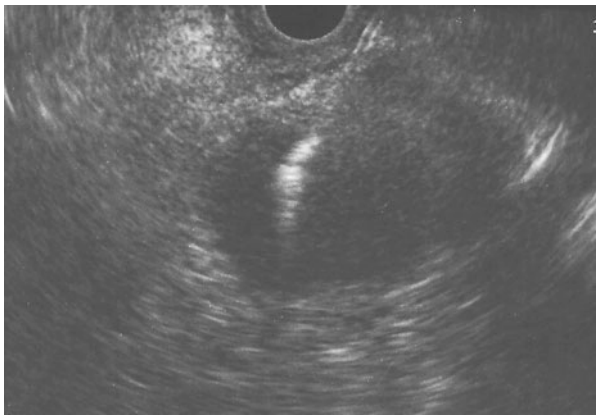

Fig. 1 EUS-FNA with a 19G needle of an echo-poor tumor in the tail of the pancreas in a 48-year-old male patient. Histology showed a poorly differentiated pancreatic carcinoma

with a 22G needle cytological approach with a separate cohort of 130 specimens that were immediately formalin fixed to preserve microcores of tissue prior to routine histological processing [38]. This study found that direct formalin fixation significantly shortened the time required for diagnosis from 3.6 to 2.9 days ($p < 0.05$) by reducing the average time (140 vs. 33 min/case) and number of slides (9.65 vs. 4.67 slides/case) for histopathological processing. Specificity and sensitivity yielded comparable results between the two approaches (82.3% vs. 77% and 90.9% vs. 100%). Importantly, EUS-FNA histology preserved the tumor tissue architecture with neoplastic glands embedded in stroma in 67.89% of diagnostic cases compared to 27.55% with the standard cytological approach ($p < 0.001$). Furthermore, microcore samples were suitable for molecular studies including the immunohistochemical and mRNA analyses. This novel approach is suggested to be suitable for future investigational trials in pancreatic cancer patients, e.g., to obtain predictive signatures prior to a planned neoadjuvant treatment.

Another fascinating and highly promising field is the use of molecular markers, e.g., DNA-analysis or genetic analysis and quantitative studies of oncogene mutations (e.g., K-ras) in specimens obtained from EUS-FNA to differentiate malignant and benign pancreatic masses, to increase accuracy of early diagnosis or to assess prognosis of pancreatic cancer patients. Various molecular markers have been considered useful and have been tried. In a study by Tada et al. [39], the combination of cytology and analysis for mutant K-ras was shown to improve diagnostic accuracy, as cytological diagnosis of malignancy by EUS-FNA was achieved in only 62% of patients with pancreatic cancer, whereas mutant K-ras was detected in 77% of the EUS-FNA aspirates from cases with pancreatic carcinoma. When cytology and K-ras mutation detection were combined, the diagnostic accuracy increased to 81%. Importantly, K-ras mutations were absent in cases with a suspicious cytology from benign pancreatic lesions. Recent miRNome analyses show that PDAC and IPMN have differential miRNA profiles with respect to controls, with a large number of deregulated miRNAs shared by both neoplastic lesions. Overall, 30 miRNAs whose expression is significantly increased in PDAC and IPMN lesions were identified and validated in this study. The feasibility of detecting these miRNAs in

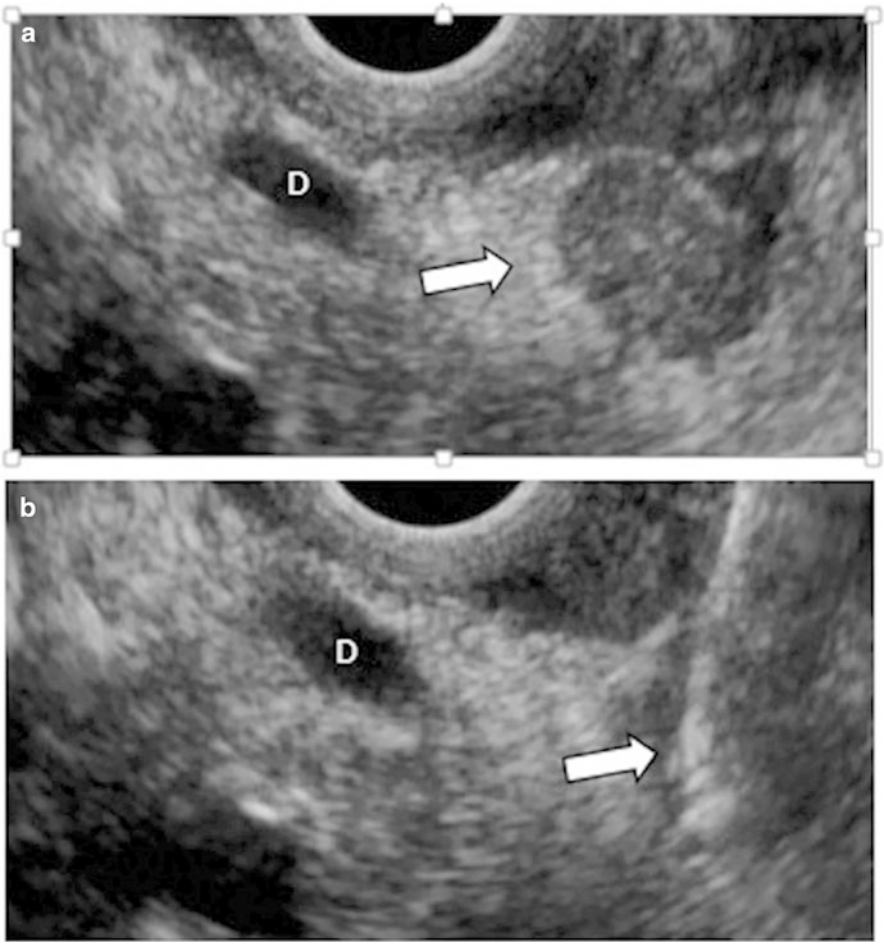

Fig. 2 (**a**) Suspected lymph node metastasis in a 56-year-old male patient with chronic pancreatitis and a mass in the head of the pancreas (D pancreatic duct, *arrow* peripancreatic lymph node) (**b**) EUS-FNA of peripancreatic lymph node (D pancreatic duct, *arrow* needle within lymph node). Histology revealed chronic inflammation but no sign of malignancy

endoscopic ultrasound-guided fine-needle aspiration samples make them good biomarker candidates for early detection of pancreatic cancer [40]. A second study demonstrated that a 2-miRNA classifier (miR-21 + miR-155) was capable of distinguishing benign from malignant pancreatic lesions in EUS-FNAs with a sensitivity of 81.5% and a specificity of 85.7% (AUC 0.930) [41]. EUS-FNA cytology genotyping using next-generation sequencing of a 160 cancer gene panel revealed a broad spectrum of pathogenic alterations that showed a high degree of concordance to paired surgical resection specimens. This fidelity suggests that sequencing analysis of gene panels in EUS-FNA may be used for molecular stratification of pancreatic tumors as the basis to personalize therapeutic decisions [42]. Yet, most of these

studies are still based on relatively small numbers of patients and on research conditions and are not yet widely applicable. Thus, encouraging and fascinating these and similar results cannot be extrapolated to everyday clinical practice yet and require further validation by studies incorporating larger patient numbers.

EUS for Cystic Pancreatic Neoplasias (CPNs)

Pancreatic cysts detected by imaging in asymptomatic patients may correspond to a variety of pathologies ranging from benign cysts (pseudocysts, serous cystic adenomas (SCA), true cysts) and premalignant or malignant cystic neoplasias (mucinous cystic neoplasms (MCNs), intraductal papillary mucinous neoplasms (IPMNs), solid pseudopapillary neoplasms (SPN), cystic pancreatic neuroendocrine neoplasias (cpNEN), serous cystadenocarcinomas). However, since up to 50–60% of the incidental pancreatic cysts detected by imaging show connections to pancreatic duct, they most likely represent intraductal papillary mucinous neoplasms (IPMNs), although there is no firm pathology to support this.

Several EUS findings have been evaluated as diagnostic criteria for pancreatic cystic lesions [43]. When surgical histology is used as a reference standard, the diagnostic accuracy of EUS imaging ranges from 40% to 96%. In a prospective study, the overall accuracy of EUS morphology for differentiating mucinous cysts (MCNs and IPMNs) from nonmucinous cysts was low (51%) [44]. In addition EUS imaging cannot reliably distinguish benign from malignant IPMNs; however, EUS may be useful in identifying predictors for malignancy. A meta-analysis including 1,373 patients found that a mural nodule, main pancreatic duct dilation, thickened septal walls, and cyst size >3 cm on radiologic or EUS imaging were independent predictors of malignant branch-duct IPMN [45]. Recently, an international consensus guideline developed in Fukuoka (Japan) identified a main pancreatic duct (MPD) size >10 mm or the presence of an enhancing solid component on radiologic imaging as high-risk stigmata (HR) [14]. Worrisome features (WF), thought to be associated with lower risk, included a cyst size of >3 cm, thickened enhancing cyst walls, nonenhancing mural nodules, MPD size of 5–9 mm, an abrupt change in the MPD caliber with upstream pancreatic atrophy, or the presence of peripancreatic lymphadenopathy (see Table 1). HR cysts are recommended to undergo surgery because of high cancer prevalence, for WFs cysts endoscopic ultrasonography (EUS) eventually with EUS-FNA for further risk stratification, and for non-HR/non-WF cysts cysts periodic surveillance at various intervals are recommended.

In the event that the combination of all available imaging modalities including contrast-enhanced MD-CT, MRI, and MRCP with diffusion weighting and EUS do not clarify the diagnosis EUS-guided aspiration of cyst fluid may help in establishing the nature of the cystic lesion. Cyst fluid sampled by EUS-FNA may be analyzed for cytologic, chemical, and/or molecular studies. Malignancy within a cystic neoplasm can be identified by cytology with 83–99% specificity, although reported sensitivities vary from 25% to 88% as summarized in [43]. Thus, a negative cytology does not help in the decision whether a pancreatic cyst is malignant or not. In addition to

Table 1 Risk stratification of branch-duct IPMN according to Fukooka guidelines

High-risk stigmata	Obstructive jaundice and cystic lesion in head of pancreas
	Enhancing solid component within cyst
	Main pancreatic duct ≥10 mm
Worrisome features	Clinical: presence of pancreatitis
	Imaging:
	Cyst ≥3 cm
	Thickened/enhancing cyst walls
	Main duct 5–9 mm
	Nonenhancing mural node
	Abrupt change in caliber of pancreatic duct with distal pancreatic atrophy

cytology, cyst fluid is analyzed routinely for amylase levels and the tumor marker CEA. These analyses do not allow to identify malignant cysts but they may help to differentiate mucinous cysts from serous cysts or pseudocysts. Studies indicate that amylase levels <250 U/L virtually exclude a pseudocyst (specificity 98%) [46] and a CEA cutoff of 192 ng/mL differentiates mucinous from nonmucinous cysts, providing a sensitivity of 75% and a specificity of 84% [44]. This is highly relevant for clinical management since nonmucinous cysts such as serous cystic adenomas have virtually no risk of malignancy and do need a less intense and no follow-up if stable after an initial follow-up period [47]. In contrast mucinous lesions such as MCN and IPMN have an inherent risk of malignancy and thus either need to be followed up more closely or resected when risk signs are present [14, 48].

In a recent a multicenter, retrospective study of 130 patients with resected pancreatic cystic neoplasms cyst fluid was analyzed to identify subtle mutations in genes known to be mutated in pancreatic cysts (BRAF, CDKN2A, CTNNB1, GNAS, KRAS, NRAS, PIK3CA, RNF43, SMAD4, TP53, and VHL) [49]. With this combined analyses, the authors identified molecular markers and clinical features that classified cyst type with 90–100% sensitivity and 92–98% specificity. The molecular marker panel correctly identified 67 of the 74 patients who did not require surgery and thereby reduced the number of unnecessary operations by 91%. In a recent study next-generation sequencing was most valuable in identifying mucinous cysts with nonmucinous CEA levels in cyst fluid FNA [50]. Molecular analysis of pancreatic cyst fluid sampled by EUS-FNA will most likely form part of the routine evaluation of pancreatic cysts, in particular, of those where size or risk features would lead physicians to recommend surgical resection.

An intriguing new approach is the development of a needle-based confocal laser endomicroscopy. A small probe can be advanced through a 19-gauge needle directly into the tissue or lesion of interest. A pilot study in pancreatic cystic neoplasms revealed a high specificity (100%) while sensitivity was low (59%) [51]. Another pilot study used this device to identify a vascular network pattern characteristic for serous cystic neoplasms [52]. Further validation of this technique will be necessary before it can be introduced in clinical practice (Figs. 3–5).

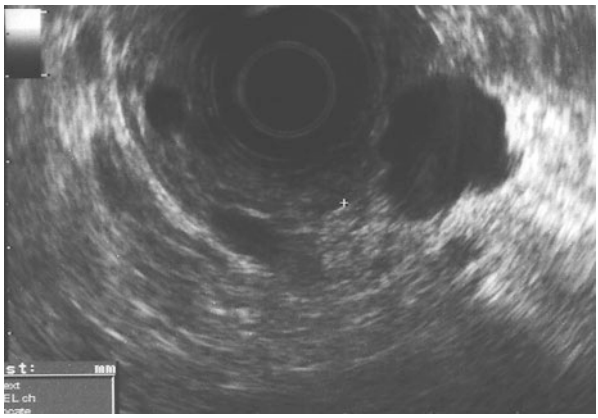

Fig. 3 Mainduct-IPMN in the tail of the pancreas in a 65-year-old female patient. Histology after distal pancreatic resection demonstrated an IPMN without signs of invasiveness

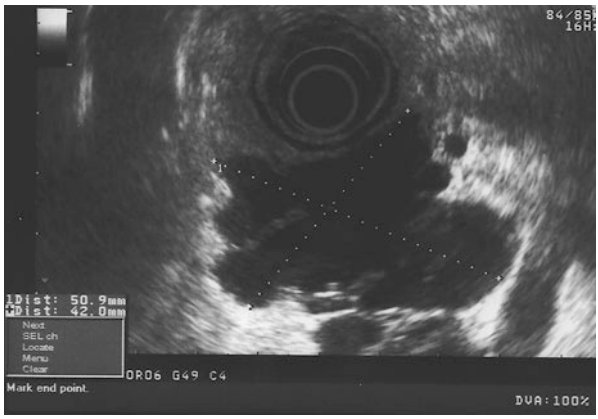

Fig. 4 Suspected serous cystadenoma in the head of the pancreas in a 77-year-old male patient

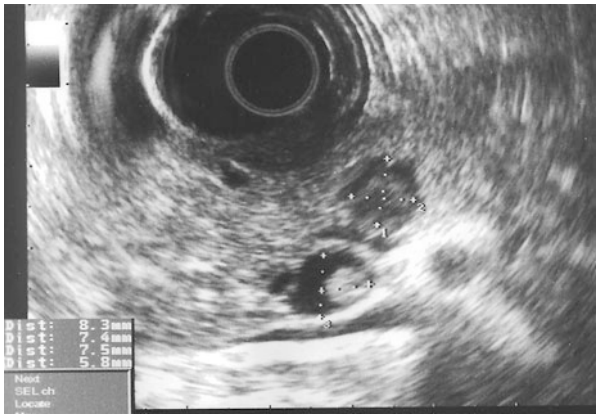

Fig. 5 Multiple branch-duct-IPMNs in a 68-year-old female patient. EUS showed several lesions with mucin but also mural nodules (worrisome features)

Screening of Individuals at Risk for Familial Pancreatic Cancer

Recent expert consensus conferences [53] considered it appropriate to perform pancreatic cancer (PC) screening in high-risk individuals for familial pancreatic cancer using a multidisciplinary approach under research protocol conditions. However, neither biomarkers nor reliable imaging modalities for the detection of high-grade precursor lesions are yet available. Most screening programs are currently based on EUS and magnetic resonance imaging, and first data demonstrated that PC precursor lesions such as IPMNs can be identified [53, 54]. There is yet no consensus regarding the age to initiate or stop screening and the optimal intervals for follow-up [53, 55–57]. A recent multicentric study could demonstrate that surveillance in high-risk individuals for pancreatic cancer carrying CDKN2A mutations was relatively successful, detecting the majority of PDACs in a resectable stage. In 13 of 178 screened high-risk individuals pancreatic adenocarcinoma was detected that could be resected in 75% [57]. In contrast, the value of surveillance for non-CDKN2 familial pancreatic cancer (FPC) family members is still not clear, and the main effect seems to be prevention of PDAC by removal of preneoplastic lesions such as IPMNs, which belong to the pancreatic phenotype of high-risk individuals for familial pancreatic cancer [54]. With further improvements in MRI technology and its wide availability, EUS may turn out to be an additional tool rather than the main test in surveillance programs. Envisaged applications for EUS are to supplement MRI combined with MRCP in longer time intervals and for specific tasks, such as the high resolution imaging of cystic pancreatic lesions. A recent multicentric study reached the conclusion that non-CDKN2A high-risk individuals for familial pancreatic cancer should receive annual MRI-based screening, starting at the age of 50, supplemented by EUS at the baseline examination. If unremarkable at baseline, the authors recommend to perform EUS only every 3 years in addition to MRI or when changes become evident in the annual MRI, eventually combined with EUS-FNA [56].

EUS-Guided Therapy: Overview and Perspectives

EUS has not only broadened its diagnostic spectrum by the use of EUS-FNA but has also entered the area of endoscopic therapy injected intra- or peripancreatically using EUS guidance.

EUS-CPN

EUS-guided celiac plexus neurolysis (CPN) in patients with refractory abdominal pain due to pancreatic cancer may be an option that has been described in detail elsewhere [58]. Advancing the needle via a transgastric approach, EUS-CPN aims to ablate the neurons of the celiac ganglia through the injection of cytolytic agents such as alcohol or phenol with prior injection of a local anesthetic (e.g., bupivacaine). Usually more than one session is necessary to achieve effective and persistent pain

relief. In an initial randomized study that compared EUS- and CT-guided CPN, the EUS approach was found to be superior [59].

A double-blind, controlled trial found that early EUS-CPN reduces pain and may moderate morphine consumption in patients with newly diagnosed, painful, inoperable pancreatic cancer [60].

The most common complications of EUS-guided CPB and CPN include transient diarrhea, pain, and hypotension, which are usually self-limiting. Gastroparesis, retroperitoneal hemorrhage, and peripancreatic abscess are rarely reported complications. However, serious adverse events include paralysis after infection of the anterior spinal cord [61], gastric perforation due to necrosis after multiple procedures [62], and infarction due to celiac artery thrombosis [63].

EUS-Guided Radiotherapy

EUS-guided fiducial placement has been used to aid in image-guided radiation therapy. Fiducials can be placed with either 19-gauge or 22-gauge needles using a technique comparable to EUS-guided FNA with or without fluoroscopy [64]. The rate of adverse events from fiducial placement is comparable to that of EUS-FNA of the pancreas. Adverse events include mild pancreatitis, minor bleeding, and fiducial migration, requiring a repeat procedure [65]. Alternatively, intratumoral radioactive seed implantation in combination with chemotherapy has been studied in 22 cases with three partial remissions and stable diseases reported. However, cancer progressed in 20 patients, all of whom died during 2 years of follow-up [66]. An additional study could not show any significant survival benefit by combining EUS-brachytherapy with gemcitabine-based chemotherapy, though a significant improvement in pain control was observed [67].

EUS-Guided Application of Immunotherapy

An allogeneic mixed lymphocyte culture (cytoimplant) delivered by endoscopic ultrasound-guided fine-needle injection in patients with advanced pancreatic carcinoma supposed to activate the host immune system was tested in a phase I trial in eight patients. Two partial and one minor response were observed with no major complications [68]. A pilot study evaluated the potential of EUS-guided injection of dendritic cells [69]. Only five patients were included yielding mixed results, though no adverse events were observed. EUS-guided transgastric/transduodenal or percutaneous intratumoral injection of tumor necrosis factor biological (TNFerade) in combination with chemoradition was tested in a large randomized trial with 304 patients with locally advanced tumors. Injection appeared safe but did not prolong survival as compared to chemoradiation alone [70]. Surprisingly, multivariate analysis showed that TNFerade injection by an endoscopic ultrasound-guided transgastric/transduodenal approach rather than a percutaneous transabdominal approach was a risk factor for inferior PFS (HR, 2.08; 95% CI, 1.06 to 4.06; $P = 0.032$).

EUS-Guided Biliary and Gastric Drainage

The use of EUS to guide biliary drainage has become an option when access to the bile duct via ERCP is not possible. Several techniques have evolved in recent years among others comprising EUS-guided guidewire placement into the common bile duct using EUSguidance and then passed through the papilla to guide further ERCP interventions as well as the direct EUS-guided transgastric or transduodenal puncture and stent placement into the common bile duct. Studies have reported high technical and clinical success rates in almost 90% of cases, but also adverse events in 10–20% [71, 72] including stent migration, bile leak, biliary peritonitis, and pneumoperitoneum.

There are limitations to enteral self-expandable metal stents and surgical gastrojejunostomy in the treatment of patients with gastric outlet obstruction (GOO). EUS-guided gastroenterostomy (EUS-GE) inserting a lumen-apposing metal stent in an adjacent jejunal loop under EUS guidance is a novel procedure that potentially offers long-lasting luminal patency without the risk of tumor ingrowth and/or overgrowth while avoiding the morbidity of a surgical procedure. In the small patient series published to date, technical success rates up to 90–100% with no or only mild procedure-related adverse events have been reported [73, 74].

Endoscopic Treatment of Cystic Lesions

Recently, endoscopic cyst ablation with ethanol alone or in combination with paclitaxel for suspected pancreatic cystic neoplasms has been proposed as an alternative to surgery [75, 76]. Since the procedure is associated with significant side effects, uncertainties remain regarding the durability of the approach, and it is unclear whether patients remain at risk to develop pancreatic cancer after cyst ablation; EUS-guided cyst ablation is not recommended as routine intervention. It should be reserved for individual centers performing this procedure using research protocols and for patients who have high-risk lesion and are not candidates for surgery [43].

Conclusion

In experienced hands EUS is the most sensitive imaging modality for pancreatic tumors <2 cm, the major limitation being its operator dependence. It has a role for preoperative pancreatic cancer staging and appears to be most useful as adjunct when staging is inconclusive in CT. Additional useful applications include the differential diagnosis of solid and cystic pancreatic lesions, rendering EUS an important baseline and adjunct screening tool in MRI-based research screening protocols for high-risk individuals for familial pancreatic cancer. EUS-guided tissue acquisition either as FNA or FNB has been established as an essential tool for differential diagnosis of pancreatic masses and cystic pancreatic lesions that is

associated with low overall complication rates. The use of EUS-FNA/FNB material for molecular analyses will expand the possible applications, e.g., for the molecular stratification of cystic pancreatic lesions or for the prognostic and predictive stratification of pancreatic cancer. EUS-guided therapeutic applications have been developed, and in particular the use of EUS to guide celiac plexus neurolysis (CPN) or biliary drainage is being used in everyday clinical practice. Overall, EUS has evolved as indispensable imaging modality for the diagnosis, staging, and screening of pancreatic cancer and its precursor lesions, with exiting novel diagnostic and therapeutic applications.

Cross-References

- ▶ Clinical Decision-Making in Pancreatic Cancer
- ▶ Management of Cystic Neoplasms of the Pancreas Including IPMNs
- ▶ MRI and MRCP for Diagnosis and Staging of Pancreatic Cancer
- ▶ Pancreatic Adenocarcinoma: CT and PET/CT
- ▶ Secondary Screening for Inherited Pancreatic Ductal Adenocarcinoma
- ▶ Therapeutic Endoscopy in the Management of Pancreatic Cancer

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Laparoscopic Staging in Patients with Newly Diagnosed Pancreatic Cancer

Timothy Gilbert, Ryan Baron, Paula Ghaneh, and Christopher Halloran

Contents

Abstract

Prompt accurate staging is paramount in managing patients with newly diagnosed pancreatic cancer. Initially, diagnosis and staging are undertaken using contrast-enhanced multidetector computerized tomography (CE-MDCT) or magnetic resonance imaging (MRI), supplemented with endoscopic ultrasound in selected cases. Staging laparoscopy (SL) with or without laparoscopic ultrasound (L-LUS) has been

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found to detect occult disease in 13–28% of patients with pancreatic cancer who are considered potentially resectable on imaging; however, between 1% and 30% of patients thought to be resectable on SL/L-LUS have subsequently been found to have unresectable disease. The clinical utility of SL/L-LUS can be enhanced by adopting a selective approach, only undertaking SL/L-LUS when one or more criteria are present, including (1) presumed pancreatic primary >3 cm diameter, (2) lesions in the body and tail of the pancreas, (3) CA 19–9>150 kU/L (>300 when total bilirubin >35 micromol/L), and (4) platelet/lymphocyte ratio >150. The judicious use of SL/L-LUS and cross-sectional imaging are complementary; however, the advent of PET-CT may lead to improvements in the detection of small previously radiologically occult metastases and may reduce the future role of SL/L-LUS.

Keywords

Pancreas · Pancreatic cancer · Laparoscopy · Laparoscopic ultrasound · Diagnosis · Staging

Introduction

It is clear that over the last 15 years, a combination of better staging, surgical refinement, and standard use of adjuvant chemotherapy has achieved an unprecedented increase in survival of patients with pancreatic cancer, who have had surgery to around 30% at 5 years [1–4]. The importance of diagnosis and staging in the management of pancreas cancer becomes evident when surveying the outcome of patients with localized versus advanced disease.

Given the marked differences in survival between those who undergo potentially curative resection compared to those who cannot, accurate selection of patients for surgery is essential. Accurate selection for potentially curative resection will ensure this is undertaken in only patients who will benefit, and major abdominal surgery avoided in the vast majority of those who will not.

A variety of imaging strategies have been studied to determine the optimal approach to diagnosis and staging of suspected pancreatic cancer [5–15]. Contrast-enhanced multidetector computerized tomography (CE-MDCT) (see chapter ▶ "Pancreatic Adenocarcinoma: CT and PET/CT"), magnetic resonance imaging (MRI with or without magnetic resonance cholangiopancreatography, MRCP) (see chapter ▶ "MRI and MRCP for Diagnosis and Staging of Pancreatic Cancer"), endoscopic ultrasound (EUS), and staging laparoscopy with or without laparoscopic ultrasound (SL/L-LUS) have all been compared, and each has their protagonists. Current recommendations [4, 16, 17] agree on a standard approach making use of abdominal imaging with CE-MDCT performed according to a defined pancreas protocol with dual arterial and portal venous contrast phases, supplemented selectively with other adjuncts including MRI/MRCP and EUS [4]. Positron emission tomography–computed tomography (PET-CT) is considered an additional diagnostic adjunct to CE-MDCT and MRI, not a substitute for these modalities [4]. SL/L-LUS is only considered a selective adjunct to diagnosis and is not routinely included in any of the current major international guidelines.

Background to Staging and Assessment by Radiological Imaging

Contrast-enhanced multidetector computerized tomography (CE-MDCT) is the "gold standard" for clinical/radiological staging, since the reported accuracy of CE-MDCT using 2D and 3D algorithms in predicting resectability can exceed 95%, with a sensitivity of 94% and a specificity of 89% [5, 7, 11, 14, 18] (see chapter ▶ "Pancreatic Adenocarcinoma: CT and PET/CT"). Resectability rates may, however, appear artificially high if surgeons adopt a more conservative approach, operating only on easy cases and do not attempt resection in borderline resectable cases. Nevertheless, in the hands of experienced pancreatic radiologists using CE-MDCT, local tumor extension, vascular involvement, and lymph node and liver metastases correlate closely with surgical findings [14].

MRI using ultrahigh-field magnetic resonance has been reported to be superior to CT in the detection of non-contour-deforming masses (small pancreas cancers) due to its superior soft tissue contrast [6, 8] (see chapter ▶ "MRI and MRCP for Diagnosis and Staging of Pancreatic Cancer"). MRI may also be preferable for characterizing small liver and peritoneal/omental metastases [6]. MRI, MRI spectroscopy, and MRI functional imaging are under development to distinguish malignant from benign pancreas tumors, using protocols based on signal intensity [15], but these techniques are yet to gain a place in optimal standard staging approaches.

EUS with or without fine needle aspiration (FNA) biopsy has been found in one study to be highly accurate in diagnosing pancreas cancer (99%) with 88% sensitivity, 100% specificity, 100% PPV, and 99% NPV in patients with ambiguous CT findings [9]. These impressive results, however, were retrospective, and surgical confirmation of diagnoses was available in only a small proportion of these patients. EUS has the advantage of enabling biopsy, but a negative FNA does not exclude cancer, and the approach is highly operator dependent. Although EUS is the preferred biopsy route rather than percutaneous image-guided approaches, a decision to operate does not require histological confirmation, although this is required prior to administration of neoadjuvant or palliative chemotherapy [4, 19].

Positron emission tomography–computed tomography (PET-CT) (see chapter ▶ "Pancreatic Adenocarcinoma: CT and PET/CT") has recently emerged as a new imaging modality in pancreatic cancer. PET-CT is found to have a similar sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) in evaluating primary tumors as CE-MDCT [10, 20, 21], EUS [13, 22], and MRI [20], indicating that PET-CT does not add to the determination of resectability of local disease. However, the majority of this evidence is from small, single-center retrospective studies. The true value of PET-CT may lie in its ability to detect metastatic disease, with studies showing it to have a much greater sensitivity than MDCT or MRI, altering proposed surgical management in 10–45% of patients deemed resectable post MDCT/MRI [20, 23, 24]. In addition it has significant advantage in diagnosing invasive transformation within premalignant lesions [25], such as intraductal papillary mucinous neoplasms (see chapter ▶ "Management of Cystic Neoplasms of the Pancreas Including IPMNs"). The

full published results of the multicenter prospective PET-PANC trial are awaited; however, preliminary data demonstrates significantly improved sensitivity (92.7% vs. 88.5%, $p = 0.010$) and specificity (75.8% vs. 70.6%, $p = 0.023$) of FDG PET-CT over MDCT in diagnosing pancreatic cancer. FDG PET-CT correctly changed staging in 14% of patients and influenced the management of 45% of patients in the trial, importantly preventing futile attempted resection in 20% of patients due to undergo surgery [26].

Despite these significant advances in imaging techniques, even with state-of-the-art machines, metastatic lesions <5 mm may still not be detected, as is often the case in small hepatic and peritoneal deposits. Presence of these deposits would likely render the otherwise radiologically resectable or borderline resectable patient unresectable and thus preclude the need for an ultimately futile laparotomy. This has a cost benefit but more importantly a benefit to the patient allowing prompt initiation of alternative treatment pathways, i.e., neoadjuvant or palliative treatment. SL/L-LUS as an adjunct to radiological staging enables direct visualization of the peritoneal cavity thus providing an opportunity to identify these small lesions and simultaneously assess local resectability particularly with respect to vascular structures. The rationale for SL/L-LUS is that it enables (1) confirmation of diagnosis when in doubt; (2) the detection of radiological occult metastasis including biopsy of suspicious lesions; (3) assessment of local resectability and (4) peritoneal cytology; it aims to prevent unnecessary operations which (5) decrease patient morbidity; (6) it enables prompt initiation of more appropriate treatment pathway, i.e., chemotherapy; and (7) it provides more cost-effective/patient acceptable disease management.

SL/L-LUS in Potentially Resectable Patients

As is implicit in the discussion above, SL/L-LUS is an aid to diagnosis and staging, but not a sine qua non. It must be remembered that laparoscopy is an invasive procedure requiring general anesthetic and the relative absence of adhesions from prior disease or interventions to fully inspect the peritoneal cavity. Even then the view of the peritoneum is an extensive sampling rather than a complete inspection, and as regards the liver, small metastases (5 mm diameter or less) are only likely to be identified on the capsular surface. Larger liver metastases can be identified with a laparoscopic ultrasound (LUS) probe, an examination which requires gentle, systematic, and complete liver scanning; although, larger metastases should be identified preoperatively by an up-to-date CE-MDCT or MRI. In addition, LUS can be used as an adjunct when assessing local respectability by helping to delineate vessel encroachment. The guidelines published by the British Society of Gastroenterology and other UK specialist societies in 2005 recommend that when available, SL/L-LUS may be appropriate in selected patients with pancreas and periampullary cancer (recommendation grade B) [27], although the practice is not yet generally incorporated in other international guidelines [4, 16, 17]. SL/L-LUS has been found in studies, from specialist pancreatic centers, to identify occult advanced and metastatic

disease in 13–58% of patients considered resectable on radiological grounds; the majority of failures to detect occult disease are due to failure to appreciate fully the degree of vascular involvement in locally advanced cases rather than missed liver or peritoneal metastases (see Table 1). Most of these studies are highly selected and designed to answer specific questions: role of preoperative cancer antigen 19-9/sialylated Lewis (a) antigen (CA19-9) (see chapter ▶ "Development of Novel Diagnostic Pancreatic Tumor Biomarkers") in selection of patients for staging [28, 29], preoperative inflammatory markers [30], subsets of peripancreatic cancers [31], or cost-effectiveness [32]. To date there are no randomized clinical trials looking at the use of laparoscopy. There has been one meta-analysis and three systematic reviews reviewing the role of laparoscopy following imaging for "resectable" pancreatic cancer. Hariharan et al. in 2010 [33] looked at the benefit of SL/L-LUS in 2827 patients across 22 studies with radiologically resectable pancreatic/peri-pancreatic cancer. Results from this analysis showed the pooled sensitivity and specificity of SL/L-LUS for the detection of liver and peritoneal lesions to be 88% (95% CI 83–92) and 92% (95% CI 84–96), respectively. However, sensitivity for detection of locally advanced disease was poor: 58% (95% CI 51–65). The pooled yield of SL/L-LUS, i.e., proportion of patients in whom unnecessary laparotomy was avoided, was 25%. A Cochrane review, undertaken by Allen et al. in 2013, reported similar results [34]. This included 15 studies with a total of 1015 patients diagnosed with resectable pancreatic/periampullary cancer following initial staging CT scan. They reported a pooled sensitivity for SL/L-LUS of 68.7% (95% CI 54.3–80.2%). From the included studies, the authors calculated a median pretest probability for unresectable disease of 0.403. This would equate to 23% of patients avoiding an unnecessary laparotomy post SL/L-LUS [34]. The authors recognized the potential impact of advances in CT scan technology and adjusted for this by performing a post hoc meta-regression of studies published before and after the year 2000 and found no statistically significant difference. This was reviewed by the same group again in 2016 [48], with 16 studies, confirming a similar result (avoidance of 21 unnecessary laparotomies). Levy et al. in 2016 [49] performed a systematic review of prospectively conducted studies assessing the accuracy of SL/L-LUS in assessing the resectability of pancreatic tumors, comparing the predicted resection rates of SL/L-LUS with standard preoperative imaging and determining how the accuracy of these modalities has evolved over time. Nineteen prospective studies met the inclusion criteria including 1573 patients; 11 of these studies were performed after January 2000 in the MDCT era. Overall SL/L-LUS improved the resection rate of pancreatic malignancies from 55% to 79% over standard preoperative imaging, preventing non-curative laparotomy in 33% of study patients, with no increase in mortality and only a 0.8% complication rate. The added benefit of LUS to staging laparoscopy was directly addressed in three studies [50–52], which collectively showed a doubling of the yield of unresectable disease versus non-ultrasound laparoscopy alone.

Subgroup analysis of more recent studies (2009–2014), studies post January 2000, and studies comparing only MDCT imaging all demonstrated comparable findings with resection rates of 100% and 81% (two studies), 74% and 58% (four

Table 1 Identification of metastatic disease with SL/L-LUS in patients considered potentially resectable on radiological grounds

Study	Technique	Resectable patients (imaging)	Patients undergoing L-LUS	Patients unresectable on L-LUS (%)	Patients undergoing surgical exploration following L-LUS (%)	Non-resected (missed occult disease) following L-LUS (%)	Patients who underwent resection following L-LUS (%)
Taylor et al. 2001 [35]	L-LUS	51	51	21 (41%)	24 (47%)	2 (4%)	20 (39%)
Menack et al. 2001 [36]	L-LUS	27	27	7 (26%)	20 (74%)	2 (7%)	18 (67%)
Vollmer et al. 2002 [31]	L-LUS	157	153	37 (24%)	–	–	–
Nieveen et al. 2003 [37]	L-LUS	297	286	39 (13.6%)	Resectable: 197 (69%)	Resectable: 52 (18%)	Resectable: 145 (51%)
					Borderline: 31 (11%)	Borderline: 20 (7%)	Borderline: 11 (4%)
Doran et al. 2004 [38]	L-LUS	190	190	28 (15%)	158 (83%)	33 (17%)	127 (67%)
Thomson et al. 2006 [39]	L-LUS	154	152	56 (37%)	87 (57%)	25 (16%)	62 (41%)
Doucas et al. 2007 [40]	L-LUS	75	75	28 (37%)	37 (49%)	22 (29%)	15 (20%)
Halloran et al. 2008 [28]	L-LUS	164	70	9 (13%)	Resectable: 37 (53%)	Resectable: 7 (10%)	Resectable: 30 (43%)
					Borderline: 24 (34%)	Borderline: 17 (24%)	Borderline: 7 (10%)

Ahmed et al. 2006 [41]	L	59	37	9 (24%)	28 (76%)	4 (11%)	24 (65%)
White et al. 2008 [42]	L	1045	1045	145 (14%)	900 (86%)	9 (1%)	891 (85%)
Shah et al. 2008	L	88	19	9 (47%)	8 (42%)	1 (5%)	7 (37%)
Enestvedt et al. 2008 [32]	L	298	86	24 (30%)	62 (72%)	16 (19%)	46 (53%)
Contreras et al. 2009 [43]	L	77	25	7 (28%)	18 (72%)	3 (12%)	15 (60%)
Satoi et al. 2011 [29]	L	61	16	5 (31%)	–	–	11 (69%)
Lavy et al. 2012 [44]	L	52	52	5 (10%)	47 (90%)	9 (17%)	38 (73%)
Garcea et al. 2012 [30]	L	157	137	22 (16%)	–	–	–
Schnelldorfer et al. 2014 [45]	L	274	136	3 (2%)	133 (98%)	12 (9%)	–

Does not include Connor et al. 2005 [46] or Smith et al. 2008 [47] as these reports include patients included in [28, 38]

studies), and 100% and 78% (one study) for SL/L-LUS versus MDCT, respectively.

All of these reviews acknowledge significant study heterogeneity, particularly with regard to resectability criteria, requirement to offer surgery for gastric outlet obstruction prior to routine use of duodenal stenting, multimodal imaging protocols, and the quality of CT technology.

Selective Criteria for SL/L-LUS

The advent of the MDCT era and more accurate preoperative imaging assessment of resectability results in a larger number of SL/L-LUS required to be performed to prevent one unnecessary laparotomy; Friess et al. demonstrate that only one laparotomy is avoided for every eight laparoscopies performed in patients with pancreatic cancer resulting in a reduction in the cost-benefit relationship associated with SL/L-LUS [53, 54]. These findings led to questioning of the clinical utility of SL/L-LUS on a routine basis and suggested a move toward selective SL/L-LUS. In addition to equivocal radiological staging, proposed criteria on which to select patients for SL/L-LUS include tumor size and tumor location, with clinical and laboratory findings associated with risk of locally advanced disease or metastasis such as hypoalbuminemia, weight loss, raised CA19-9, and back pain [55].

CA19-9

Early work by Doran et al. (2004) found SL/L-LUS to correctly identify unresectability in 28 (15%) of 190 patients considered potentially resectable on radiological (CE-MDCT) grounds [38]. Subsequent work by Connor et al. (2005) suggested that the utility of SL/L-LUS could be improved to detect unresectability in 20/78 (25%) of those considered potentially resectable, by selecting only those for SL/L-LUS with elevated CA19-9 levels above 150 kU/L or above 300 kU/L in the presence of an elevated serum bilirubin (>35 micromol/L, to account for the effect of cholestasis) [46]. This strategy was tested prospectively in a cohort of 164 [28] subsequent patients with potentially resectable disease on CE-MDCT. Ninety-four patients (including 14 who had gastric outlet obstruction and a high CA19-9, who would need surgery regardless) went straight to surgery. Sixty-three of the 80 (79%) patients with low CA19-9 were resected versus 2/14 (14%) with high CA19-9 and symptoms. Alternately, 70 patients went to L-LUS; this included 55 patients with high CA19-9 *and* 15 patients with low CA19-9 but with suspicious CT features. Nine patients (13%) were unresectable on L-LUS (one patient with low CA19-9). Thirty-seven patients were considered resectable of whom 30/37 (80%) were resected, 28 with a high CA19-9 and 4 with a low CA19-9. The other 24 patients were thought to have features of borderline respectability (notably vascular contact/distortion); 7/24 (29%) were resected, 5 with a high CA19-9 and 2 with a low CA19-9. The sensitivity of L-LUS for detecting unresectable disease in patients with a high CA19-9 level was 33%. This assumed that all borderline disease seen on L-LUS was resectable ($P < 0.001$). This remained the case even when borderline operable L-LUS disease

was assumed to be inoperable, in which case the sensitivity, specificity, positive predictive value, negative predictive value, and accuracy for L-LUS in detecting unresectable disease became 52%, 93%, 79%, 79%, and 79%, respectively ($P < 0.001$) [28]. These findings are supported by data from the Memorial Sloan-Kettering Cancer Center (MSKCC): in 262 patients with radiologically resectable pancreatic cancer, preoperative CA19-9 >130 U/ml was strongly associated with the identification of unresectable disease (HR 2.70; 95% CI 1.34–5.44; $P = 0.005$) [56].

Pancreatic Tumor Size and CA19-9

Satoi et al. selected patients for SL/L-LUS with both of the previously established risk factors for unresectable disease tumor size >3 cm [57, 58] and CA19-9 >150 U/ml [28, 46, 59]. Of 61 patients in this cohort, 16 patients underwent laparoscopy, 5 (31%) of which were unresectable. The remaining 11 patients were all resected. Only 4.4% of patients who did not meet the criteria for laparoscopy and went straight to laparotomy were found to have unresectable disease. The combination of tumor size >3 cm and CA19-9 > 150 U/ml was significantly associated with disease unresectability ($p = 0.0147$). The relatively high rate of vascular resection in this case series may account for the high resection rates observed with 29% of patients undergoing either portal vein or coeliac trunk resection [29].

Platelet/Lymphocyte Ratio

Smith et al. (2008) hypothetically evaluated the addition of the platelet/lymphocyte ratio to the currently used Ca19-9 selection criteria. Platelet/lymphocyte (P/L) ratio >150 was used as a marker for a pro-systemic inflammatory response associated with tumor invasiveness [47]. Based on the group of patients selected for SL/L-LUS on the basis of CA19-9 alone, they found that the addition of platelet/lymphocyte ratio >150 could improve both the sensitivity (96% vs. 51%) and positive predictive value (95% vs. 83%) of SL/L-LUS beyond that of Ca19-9 alone. This additional criterion would have reduced the number of SL/L-LUS by 21% at the expense of only a 5% false positive rate in those additional patients going straight to laparotomy, which is comparable to that seen in existing cohorts going straight to laparotomy. The combination of indices has still to be tested prospectively.

Pancreatic Tumor Location

The location of the tumor within the pancreas also affects the rate at which radiologically occult metastatic disease is identified relating to the fact that body and tail lesions usually present later due to a paucity of early symptoms compared with lesions in the pancreatic head [60, 61]. Two studies of SL/L-LUS have identified metastatic lesion twice as frequently when evaluating lesions in the body and tail of the pancreas compared with lesions in the head of the pancreas. Jimenez et al. identified metastasis in 39% of patients with body and tail lesions compared with only 17% of pancreatic head lesions [62], whereas Liu et al. found metastases in 53% of body and tail lesions and 28% of pancreatic head lesions [63]. The overall higher rate of metastasis detection by Liu et al. reflects that their population only included patients with locally advanced radiologically unresectable pancreatic cancer

patients. The utility of SL/L-LUS based on histological diagnosis has also been analyzed. Both found that the incidence of radiologically occult unresectable disease was higher for pancreatic head lesions compared with duodenal or ampullary lesions. Vollmer et al. discovered metastatic disease or local invasion of vessels precluding resection in 31% of patients with radiologically resectable pancreatic head cancers at SL/L-LUS; in contrast no patients with carcinomas of the ampulla or duodenum were discovered to have either metastatic disease or locally advanced unresectable disease as a result of SL/L-LUS [31]. White et al. confirm this observation finding unresectability in 17% of patients with potentially resectable pancreatic head adenocarcinoma imaged outside their institution and 8% of patients imaged within their institution; in contrast only 4% of patients with "non-pancreatic" tumors were found to have unresectable disease [42]. Both authors support only using SL/L-LUS in patients with pancreatic head cancers rather than peripancreatic disease; however, often a firm histological diagnosis is a retrospective finding only after the lesion has been resected and subjected to histological analysis, and therefore the clinical significance of these studies may be limited.

Shah et al. report their experience of selective use of SL/L-LUS in patients with MDCT-presumed resectable pancreatic cancer based on five criteria: primary tumor >4 cm in diameter, weight loss >20%, ascites, CA19-9 >1000 kU/L, or ambiguous findings on CE-MDCT. In their study SL/L-LUS avoided unnecessary laparotomy in 11 of 49 (22%) patients. This improved the positive predictive value of their staging protocol from 69% based on MDCT assessment alone to 89% based on MDCT and SL/L-LUS findings combined. Interestingly, 49% of patients meeting their criteria for SL/L-LUS had radiologically questionable liver lesions on MDCT, and in the current era, MRI may be a more appropriate and noninvasive modality by which to further characterize these lesions rather than SL/L-LUS.

Peritoneal Cytology at L/LUS

The value of peritoneal cytology obtained at SL/LUS for the staging of pancreatic cancer has been highlighted in work by Warshaw and colleagues at the Massachusetts General Hospital [60, 62, 64–66]. This work suggests that the presence of pancreatic adenocarcinoma cells in peritoneal ascites or irrigation fluid (undertaken with 500 ml saline) is a feature of advanced disease (M1 on the TNM system), whether or not there is other evidence of unresectability. Such a classification is consistent with the seventh edition of the American Joint Committee on Cancer (AJCC) staging system, which classifies positive peritoneal cytology as stage IV disease for pancreatic adenocarcinoma [67]. Supporting this Merchant et al. demonstrated that positive peritoneal cytology had a positive predictive value of 94%, specificity of 98%, and sensitivity of 25% for determining unresectability [68]. Although reduced overall survival associated with positive peritoneal cytology has been shown in a number of studies, median survivals are similar to that of patients with stage IV disease [69]. Yamada et al. demonstrated that resected patients with positive cytology had a significantly better survival (14.3 months) than patients with either cytology-negative or cytology-positive

unresectable disease (7.3 and 6.8 months, respectively; both <0.001). Among patients with positive cytology, median survival was longer in those who underwent adjuvant chemotherapy rather than those who underwent surgery alone (15.3 vs. 10.0 months) although this did not reach statistical significance. Positive cytology did not independently predict survival in their study [70]. The significance of positive peritoneal cytology on overall *and* disease-free survival has also been questioned in the setting of patients undergoing neoadjuvant chemotherapy prior to resection, although further research is required in this setting [71].

L/LUS in Radiologically Unresectable Patients

Many studies of the utility of SL/L-LUS have included patients with locally advanced unresectable disease [62, 63, 66, 72]. Two studies have included only patients with radiologically locally advanced unresectable disease due to vascular encasement [63, 73]. These studies found radiologically occult metastases on SL/L-LUS in 34% [63] and 37% [73] of patients. This distinction is clinically important in centers where patients with metastatic disease receive chemotherapy, whereas those with locally advanced unresectable disease in the absence of metastases receive chemoradiotherapy. By diagnosing radiologically occult metastatic disease, patients who will not benefit from chemoradiotherapy are spared the additional toxicity and time expenses associated with this therapy. On a population level, correctly staging patients to stage IV disease rather than stage III disease allows a better understanding of treatment protocols and stage-specific survival [73, 74].

Cost-Effectiveness of SL/LUS

An important issue in SL/L-LUS is its operational effectiveness, not least of which is cost. A cost study from the USA found that the use of SL/L-LUS in patients with pancreatic cancer does not add significantly to the overall expense of management: the cost for selective, routine use, or no use was found to be $91,805, $90,888, and $93,134, respectively [32]. By using pre- and posttest probabilities for unresectability [34], a UK study developed a model-based cost analysis for SL/L-LUS in pancreatic cancer [75]. Results of this analysis showed that laparoscopy prior to resection incurred similar cost per patient as proceeding straight to laparotomy, with the cost of the laparoscopy (£995) being offset by the savings of an unnecessary laparotomy (£7470; 95% CI £7215 - £7724 vs. £7480 95% CI £7219- £7741). Although, this was only the case if laparoscopy was performed at a separate sitting to the intended laparotomy as a positive SL/L-LUS conducted immediately prior to the intended laparotomy would result in a canceled operation and thus wasted theater resources. More importantly, however, this study showed that the quality adjusted life years (QALYs) were higher for SL/L-LUS compared to direct laparotomy (mean QALYs per patient 0.346 (95% CI 0.346–0.347) versus 0.337 (95% CI 0.337–0.338)) due to the morbidity associated with an unnecessary laparotomy [75]. A similar model-based cost analysis using published data on

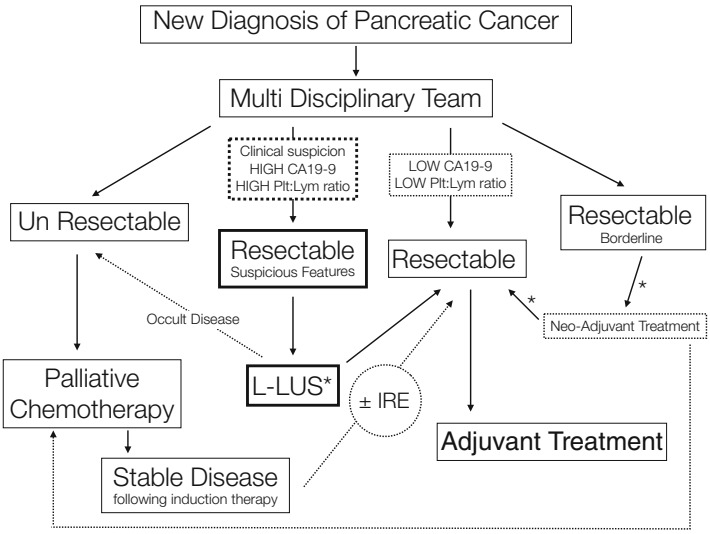

Fig. 1 Outlines a general algorithm to the management of pancreatic cancer, including selective application of L/LUS. *L-LUS* laparoscopy with laparoscopic ultrasound, *CA19-9* cancer antigen 19-9, *Plt: Lym ratio* platelet: lymphocyte ratio, *IRE* irreversible electroporation "Nanoknife™"

unresectability post-laparoscopy was conducted by a group in the USA [76]. In this study they also found an improvement in quality of life (QoL) when laparoscopy was performed prior to laparotomy and demonstrated a marginal cost saving (US$36,580 vs. US$46,830). As both these cost analyses rely on pooled estimates from the current literature, it's unclear whether the application of more selective criteria to patient selection of SL/L-LUS as discussed above would result in improved cost-effectiveness.

The current evidence would suggest that SL/L-LUS is at least cost neutral and appears to be associated with a slight improvement in QoL. It would therefore appear that the choice of whether to use SL/L-LUS in staging relates to other practical considerations, such as management priorities and practices, staff, and surgical and hospital resources, as well as additional uses to which laparoscopic approaches may be put to use, such as laparoscopic bypass surgery or evaluation of novel techniques or technologies (e.g., nano-device implantation). Figure 1 indicates where SL/L-LUS sits in current treatment algorithms.

Conclusion

Current imaging protocols and technology have resulted in significantly improved sensitivity and specificity for the diagnosis of locally advanced unresectable or metastatic disease. This has resulted in a reduction in the utility of SL/L-LUS, as it correctly identifies unresectable disease in only 15% of an *unselected* radiologically resectable population with pancreatic cancer. This has led to the *selective* use of SL/

L-LUS in patients considered at higher risk for metastatic or locally advanced cancers based on criteria such as tumor size and location, elevation of CA19-9, and questionable radiological findings. This selective use of SL/L-LUS has increased its positive predictive value back to 20–30%. Currently, SL/L-LUS is of greatest clinical utility in assessing for liver or peritoneal metastases (sensitivity 88% and 92%, respectively) and more limited in assessing locally advanced disease with vascular involvement (sensitivity 58%). The future use of SL/L-LUS will have to be continually reevaluated in light of advancing imaging technology, namely, FDG PET-CT, that is shown to improve staging of patients in a large multicenter prospective trial. This improvement in staging is of the same magnitude as that seen for SL/L-LUS, and it will be interesting to see if FDG PET-CT replaces the need for SL/L-LUS or finds a complimentary role alongside SL/L-LUS especially when combined with development in novel biomarkers. Development of future laparoscopic instruments, potentially incorporating confocal probes, may lead to prospective data on regional and or distant lymph node metastases, potentially even allowing sampling of crucial groups, allowing yet further staging potential.

Cross-References

- ▶ Development of Novel Diagnostic Pancreatic Tumor Biomarkers
- ▶ Management of Cystic Neoplasms of the Pancreas Including IPMNs
- ▶ MRI and MRCP for Diagnosis and Staging of Pancreatic Cancer
- ▶ Pancreatic Adenocarcinoma: CT and PET/CT

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Palliative Management of Pancreatic Cancer

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Abstract

The results of anticancer therapy are suboptimal for pancreatic cancer and palliation of symptoms is an important goal. Pain, depression, cachexia, ascites, jaundice, thrombosis, and gastroparesis occur commonly in pancreatic cancer patients. Painless jaundice, often associated with cancer of the pancreatic head, can be surgically treated in resectable cases or managed with a biliary stent in patients with locally advanced or metastatic disease. Pain control is optimally achieved with the use of oral analgesics; however, a neurolytic celiac plexus block can be considered when oral opioids are ineffective. Depression is associated with poor symptom control, diminished social support, and advancing illness and should be treated. Symptoms of intractable nausea, early satiety, and weight loss, in the absence of mechanical gastric outlet obstruction, suggest gastroparesis. Prokinetic agents are beneficial for some patients, but in extreme cases, gastrostomy or jejunostomy is required. Cachexia is difficult to treat and requires nutritional support, orexigenic agents, diabetic control, and enzyme supplementation. Malignant ascites can be investigated with ascitic-serum albumin gradient; a high gradient in the absence of positive cytology suggests portal vein thrombosis. Constipation is common problem and can be treated with stool softeners, osmotic agents, and peripherally acting opioid receptor antagonists.

Keywords

Pancreatic cancer · Palliative care · Cancer pain · Anorexia-Cachexia syndrome · Gastroparesis · Jaundice · Depression · Ascites

Introduction

Pancreatic cancer is one of the most feared diseases by patients and families because it is associated with symptoms that are often difficult to manage and poor prognosis. Indeed, there has been disappointingly little progress in the therapy for this disease over the past 30 years. Among patients with resectable disease, who undergo surgery alone, about 10% attain long-term survival, and roughly 20% survive only with some form of adjuvant therapy [1]. In advanced disease settings, the median survival duration is approximately 6 months and 18–23% of patients who receive palliative chemotherapy survive for 1 year [2]. Most patients who experience response to chemotherapy experience disease progression within 3–4 months and develop worsening of symptoms 1–2 months prior to documented treatment failure [3].

Cancer treatment yields a clinical benefit response in roughly 20–25% of patients overall [4], but only about 10% of moderate-to-severe symptoms at baseline improve [5]. The most prevalent and bothersome symptoms for patients with pancreatic cancer are fatigue, anorexia and weight loss, abdominal pain, constipation, and sleep disturbances [6]. Jaundice and pruritis are also common; more than two-thirds of patients with pancreatic head tumors experience jaundice. Biliary drainage is a critical first step in their palliation and should precede use of palliative chemotherapy.

Principles of Palliative Care

Palliative care is often misunderstood. Cancer patients, caregivers, and even healthcare providers believe palliative care is used only when no other options remain and results in a shortening of lifespan. The World Health Organization's definition of palliative care is:

> Palliative care is an approach that improves the quality of life of patients and their families facing problems associated with life-threatening illness through the prevention and relief of suffering by means of early identification and impeccable assessment and treatment of pain and other problems, physical, psychosocial, and spiritual. [6]

Preliminary studies show earlier intervention by palliative care providers in patients with pancreatic cancer may improve QOL [7]. There are limitless methods of integrating optimal disease management and palliative care. The most appropriate plan for a given patient depends on many factors, including the trajectory of the patient's illness, the subjective experience of the symptoms, access to personal and professional care, and personal preferences.

Comprehensive Assessment

On the basis of our current understanding of symptom biology and symptom management, what does effective symptom-directed care involve? In interdisciplinary care, comprehensive assessments of the patient and family caregivers are collected and assimilated to help providers understand their sources of suffering. Appropriate interventions are implemented to reduce suffering, and most importantly, symptoms are reassessed. Assessment is not only the key to finding and solving various problems, but also a therapeutic tool in its own right. However, comprehensive assessment and treatment of a patient's symptom burden delivered in a longitudinal fashion is often challenging.

Multi-item screening tools, symptom surveys, and quality of life instruments are frequently used in research settings but are difficult to integrate into routine cancer-care practice in either academic or community settings. One commonly used pancreatic cancer-specific assessment module is the quality of life questionnaire-pancreatic

cancer module (QLQ-PAN 26), an instrument that comprises 26 questions on disease and treatment-related symptoms and emotional issues common in this disease including pain, altered bowel habits, dietary changes, jaundice, body image, sexual functioning, and emotional issues [8]. Another hepatobiliary cancer-specific module, the Functional Assessment of Cancer Therapy-Hepatobiliary (FACT-Hep), is also valid and useful, and an expert panel reduced the item pool from 26 to a final version involving 8 specific symptoms that were clinically relevant to address when treating hepatobiliary disease [9]. In nonresearch settings, pain assessment is the most useful starting point in pancreatic cancer care, and assessment of other symptoms often follows the same general model whereby the clinician ascertains the severity, location, timing, duration, precipitating factors, and relieving factors.

Establishing Goals of Care

Ms. N was a 60-year-old Caucasian woman with type II diabetes and hypertension. She experienced worsening glycemic control and weight loss for several months. Abdominal imaging revealed a large mass that infiltrated the body of the pancreas and celiac axis. Pulmonary, peritoneal, and right ovarian masses were visible and suggestive of metastatic disease. An ultrasound-guided fine needle aspirate confirmed ductal adenocarcinoma. She experienced moderate-to-severe right flank pain, chronic constipation, and early satiety.

The treatment goals in advanced pancreatic cancer care may include: cure, prolongation of life, control of symptoms, promotion of quality of life, and prevention of suffering. Some goals may be pursued simultaneously, and sometimes some of the goals may be considered conflicting. It is usually helpful to explore three fundamental questions with the patient and the family: (1) "What is happening to me?" (2) "What is going to happen?" and (3) "What can be done to help me?" The patient can be asked what his or her understanding of the diagnosis is and what it means. One might explore this by asking "Where do you see things going with your illness?" It is also worth asking about the patient's preference for information both in terms of how it is communicated and what level of detail is suitable.

Some unintentional clinician behaviors during goals of care discussions at the end of life can result in mistrust, entrenchment in pursuing futile therapies, or even requests for hastened death [10]. Examples of such unintended behaviors include:

1. Inadvertently linking relief of suffering to acceptance by patients and family of impending mortality
2. Debating with the patient and family about the reality of impending death or failing to assess their readiness to discuss the topic
3. Misunderstanding normal grief and expressions of the "wishful ideal" as denial

A metaphor that can be useful in helping clinicians communicate about the goals of care and avoid unintended behaviors is the quality of life tank model. As shown in Fig. 1, this model involves conceptualizing quality of life as a tank that can be filled

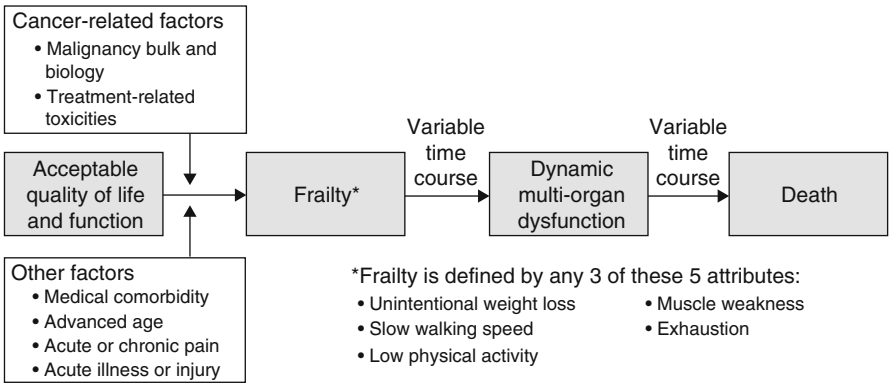

Fig. 1 The tank model

to certain levels. The very highest levels of the tank might best be considered in the realm of the "wishful ideal." For example, if Mrs. N were to talk about her deep need to help raise her 5- and 7-year-old grandchildren and see them go to college, this would be a truthful expression of her role and the perceived time horizon she once had, both of which are losses to now grieve. The clinician need not feel compelled to re-emphasize the terminal nature of the disease or assume that the patient is experiencing denial that will complicate her ongoing care. Instead, the clinician should acknowledge the patient's statements of the wishful ideal saying something like: "This is all just unspeakable. I wish things were different for you" [11]. The conversation can then transition to identifying the "realistic ideal," which is a specific level in the quality of life tank. This ideal is developed using the clinician's knowledge and experience and is often far more ambitious than what the patient actually expected. The clinician might say "Realistically, I expect that the new pain medications that I've prescribed along with the laxatives have a good chance at helping you feel much more energetic. I'd like to see you feeling well enough to be able to shop for your groceries on your own as soon as possible."

Mrs. N was anxious for detailed information and highly educated. She evaluated the risks and benefits of chemotherapy and decided to forego what she considered the small magnitude of potential benefit of systemic therapy. She focused on best supportive care alone, and sought relief of pain and constipation. When asked "What questions or concerns do you have," she indicated that it was puzzling to her how exactly pancreatic cancer causes death. Some deaths can seem rather sudden due to bleeding or infection or pulmonary embolism. On other hand, in most cases death is a slow process that occurs over a period of few months. Fig. 2 illustrates the "undertow" of advanced pancreatic cancer and the common transitions that patients make from acceptable quality of life and function to frailty, followed by multi-organ dysfunction and death. It was acknowledged that there remains uncertainty about the timing and nature of death, even when facing advanced pancreatic cancer. For this patient, who wanted information and was able to think abstractly, this model of the undertow model due to pancreatic cancer helped her feel more at ease. She strived to

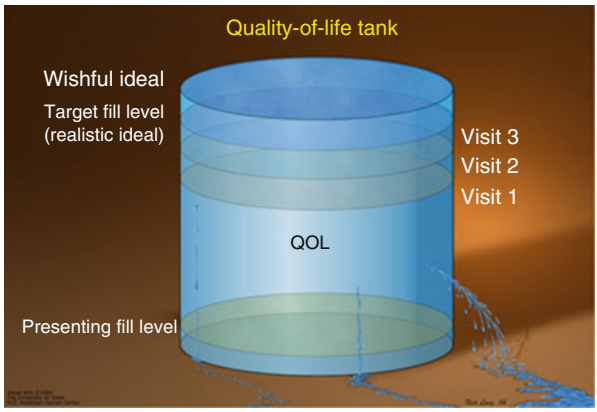

Fig. 2 The end-of-life trajectory in advanced pancreatic cancer

retain her health in the "acceptable quality of life and function" category by "paddling" against the undertow by using pain medication and laxatives, and by drawing upon her family and spiritual resources. Overall, every patient has their own way of assigning meaning and value to the potential goals of care, and no specific goal is inherently more important than another.

Systems and Teamwork

Mr. K was a 45-year-old single man with metastatic pancreatic cancer who was admitted to the hospital with abdominal discomfort, anorexia, recent weight loss, mild diarrhea with 3–4 loose stools per day, intermittent visual hallucinations, and shaking chills without fever. Three years earlier he had been treated for borderline resectable pancreatic cancer with chemoradiation followed by pancreaticoduodenectomy. He now has biopsy-proven mesenteric and retroperitoneal disease recurrence along with bilateral pleural effusions.

Pancreatic cancer care involves multidisciplinary collaboration. The multidisciplinary model is most emphasized in the initial care planning when gastroenterology, gastrointestinal medical oncology, surgical oncology, radiation oncology, pathology, and radiology are all represented in tumor board or treatment planning conferences for patients with localized pancreatic cancer. During the trajectory of illness, some of the disciplines remain closely involved, some fade back to an appropriate degree, and other services may join the team. Mr. K′s team included experts in pain management, palliative care, nutrition, and social work. During his hospital admission, many potential providers were available for problem-oriented assessment and care delivery. For most of these disciplines, the usual inpatient providers were not necessarily the same clinicians involved on an outpatient basis. Likewise, the medical disciplines also involved mid-level providers and sometimes clinicians in training. In these situations, the complexity of care can become nearly overwhelming, prone to errors, and miscommunication.

High-performing teams involved in pancreatic cancer care develop and discuss on an ongoing basis processes for specifying how work will proceed; who will do what for whom, with what purpose, when, where, and how. Multidisciplinary case conferences are not always restricted to treatment planning for new patients, but may also include some discussion of complex care for symptomatic patients who are at a different point in the trajectory of illness.

Pain Assessment and Management

Mr. D was a 52-year-old African-American man with metastatic pancreatic adenocarcinoma. His cancer was diagnosed after he had presented with persistent abdominal pain after an umbilical hernia repair. He had a 3.9 cm mass in the body of the pancreas and peritoneal carcinomatosis. On his symptom evaluation, he rated the severity of his pain in the past 24 h as an 8 on a numerical scale of 0 (the symptom not present) to 10 (the symptom is as bad as one can imagine it). He described the pain as a steady, constant feeling, similar to a heavy weight. He also experienced dysesthesias from the surgical incisions which he described as "the nerves are waking up" and a burning sensation in the periumbilical area (he was not sure whether that was acid indigestion or pain from his pancreas). Prior to his medical oncology visit, he had been prescribed 20 mg of long-acting oxycodone twice daily, but he found that to be difficult to tolerate because of a feeling of dizziness and sedation. On the remainder of his symptom inventory, he rates his fatigue at 2, nausea at 3, disturbed sleep at 3, and feelings of distress at 4. He did not experience shortness of breath, difficulty remembering things, lack of appetite, drowsiness, or dry mouth. He had no diarrhea, although he rated constipation at a 3 on a scale of 10. In terms of the way symptoms interfering with his life in the past 24 h, he rated the severity of interference in his general activity as a 7, mood as 5, work as 7, relations with other people as 5, walking as 4, and enjoyment of life as 5.

More than two-thirds of patients with pancreatic cancer experience pain at the time of their diagnosis. Pain is a particularly common presentation in patients with disease in the body or tail of the pancreas, whereas pancreatic head involvement presents as painless jaundice. Pancreatic cancer pain is often epigastric or in the central abdomen, chronic in nature. In some patients, pain may radiate to upper back or shoulder.

Successful symptom management is a multistep process, and attributions of success can be difficult. Managing symptoms, particularly pain in cancer patients, usually involves uncertainty as to the nature of the problem, and the reason for improvement. This uncertainty is partly due to the natural history of some symptoms. As in Mr. D's case, postoperative pain may have been involved, which improves over time. Interventions can reduce the severity and duration of the problems, but it is difficult to be sure how much to credit a specific intervention. In addition, there are almost always multiple simultaneous interventions. It is worth emphasizing that there is no need to delay analgesic therapy for the purpose of

investigating the cause of a complex abdominal pain syndrome in patients with pancreatic cancer.

Principles of Medical Pain Management

Mr. D's case vignette highlights several fundamental points in managing cancer pain in general, and pancreatic cancer pain in particular. First, based on the severity of the pain expression, an opioid analgesic was appropriate. If the oral route is available, as in this case, it should be used because it is effective, convenient, and cost-effective to do so. The patient's difficult initial experience with long-acting oxycodone was explicitly addressed with open-ended questions to discover his fears and misconceptions about opioids. Patients should be taught the potential benefits of opioids and the expected side effects and their management; distinctions between addiction, dependence, and tolerance should be explained.

For Mr. D, the starting opioid dose at 40 mg oxycodone per day (a morphine equivalent dose of 60 mg day^{-1}) was probably too high [15]. Dizziness and sedation, most likely due to the new opioid treatment, tend to improve spontaneously after 1–3 days with continued opioid exposure. However, patients do not accommodate to opioid side effects such as dry mouth and constipation. For that reason, it is critical to coprescribe laxative therapy along with strong opioids.

The choice of type of the initial opioid is not critical. One option would be to prescribe a short-acting, strong opioid (such as oxycodone, morphine, or hydromorphone) every 4–6 h around the clock. In this case, a low-dose sustained release opioid would also be reasonable. Examples include 20 mg day^{-1} of oxycodone, 30 mg day^{-1} of morphine sulfate, or 7.5–10 mg day^{-1} of methadone. In patients with renal insufficiency, fentanyl or methadone would be preferable long-acting opioid to minimize risk of delirium or myoclonus.

Mr. D's pain had a neuropathic component that may have been due to his recent surgery. Because he was starting an effective, tolerable opioid regimen, no specific adjuvant analgesic for neuropathic pain was needed. Opioids are effective for neuropathic pain (response rates roughly 40–50%), but that response rate is somewhat lower than for other pain syndromes such as somatic or visceral pain. There is little evidence to support the use of specific adjuvant drugs for pancreatic cancer pain management, but short-term corticosteroids and nonsteroidal anti-inflammatory agents are also particularly useful adjuvant analgesics in this disease.

The Role of Procedures Such as Neurolytic Celiac Plexus Block

The divisions of the vagus and splanchnic nerves from the celiac and mesenteric plexuses, and nerve fibers travel along the celiac and mesenteric arteries and their branches, reaching the pancreas and other viscera [12]. The celiac plexus is at least partly involved in the innervation of the pancreas, liver, gallbladder, adrenal, kidney, and gastrointestinal tract from the level of the gastro-esophageal junction to the

splenic flexure. As such, interventional pain specialists (usually trained in anesthesiology) have advocated for the use of neurolytic celiac plexus block (NCPB) on the basis of multiple uncontrolled trials and a few controlled trials [12].

The largest and most carefully conducted trial comparing NCPB with optimized systemic analgesic therapy involved the random assignment of 100 patients at a single institution with unresectable disease who received NCPB versus optimized systemic analgesic therapy plus sham injection. NCPB did not improve quality of life or overall survival in the cohort, nor did it significantly reduce opioid side effects or opioid consumption. However, NCPB did reduce the proportion of patients who experienced moderate to severe levels of pain in the first 6 weeks as compared with medical therapy (14% vs. 40%) [13]. The ideal time to consider a neurolytic celiac plexus block is unclear in patients with pancreatic cancer. In some instances, NCPB should be considered when opioid therapy is unsatisfactory or poorly tolerated, for instance, resulting in severe constipation. When palliative care expertise is available, use of opioid rotation (switching) and other assessment and treatments of not only physical but also emotional and existential pain is indicated for difficult cases. NCPB is generally a safe procedure, but the sympathetic denervation causes hypotension and hyperperistalsis (with diarrhea) in about one-third of patients, along with some local pain associated with the procedure. Major neurological complications such as paraplegia have been reported but are very rare.

Novel modalities for denervation have been used for pain resulting from pancreatitis and in some instances, from pancreatic cancer. Thoracoscopic splanchnicectomy (TS) can alleviate pain in >90% of patients with chronic pancreatitis [14]. However, the morbidity associated with this procedure is significant (16% as reported in a literature-based review) [14], as is the risk of conversion to open thoracotomy. While this procedure can be safely performed in a high-volume center, its benefit in the frail pancreatic cancer population may be outweighed by its associated risks. Small case series have also reported successful neurolysis using endoscopic ultrasound (EUS)-guided blocks [15]. EUS is now standard in the diagnostic work-up of these patients, and EUS-guided block may have potential value in patients whose pain is refractory to oral opiates [16]. However, larger randomized studies are needed to validate the use of this technique.

Depression

Mrs. L was a 57-year-old Caucasian lady old with metastatic pancreatic carcinoma with progressive disease for which she has recently started second-line combination chemotherapy. She was grieving for the loss of her mother, who had died recently of dementia. She had chronic, cancer-related abdominal, and pleuritic pain which was well-controlled with oral opioids. She appeared quite cheerful, but when asked about her mood, she admitted to feeling sad and blue. She admitted to a prior suicide attempt in early adulthood in the setting of severe depression, but she had discontinued antidepressant therapy 3 years before she being diagnosed with pancreatic cancer.

Depression is estimated to affect over 120 million persons worldwide [17]. Large, prospective studies have shown that the prevalence of major depression in the outpatient primary care setting is 6–14%, and the lifetime incidence of major depression is approximately 15% [18]. Depression is at least 2–3 times more common in hospitalized patients or patients with chronic illness [19]. Major depressive disorder is an illness that can lead to substantial morbidity due to severe functional impairment and risk of mortality because of suicide [20].

The association between pancreatic cancer and depression has been observed and explored for over 70 years. On one hand, it seems reasonable that patients with such a difficult, polysymptomatic disease would be susceptible to depressive disorders. However, Holland and colleagues found, after controlling for demographic and medical attributes, that self-ratings of depression were higher for pancreatic cancer patients than those for gastric cancer, a similarly difficult abdominal neoplasm [21]. More recently, Carney and colleagues conducted a large retrospective cohort study using longitudinal population-based insurance claims data and found that depression more commonly preceded pancreatic cancer than it did other gastrointestinal malignancies with an odds ratio of 4.6 (confidence interval 1.07–19.4) [22]. The biological basis of the relationship between pancreatic cancer and depression is not clear. The most common theories involve serotonin: this hormone may be secreted by pancreatic tumors or secreted antibodies could block the central serotonin receptors [23].

The paradigm for the diagnosis and treatment of depression is no different in pancreatic cancer than in the primary care setting. Most commonly, the diagnosis of depression is based on patient history and by the exclusion of competing diagnoses, using the criteria from the fourth edition of the *Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition* (DSM-V) [24]. Major depression is defined as depressed mood or anhedonia for at least 2 weeks plus the presence of three or four other specific psychological symptoms including feelings of guilt or worthlessness and suicidality as well as vegetative signs (sleep disorder, poor concentration, and psychomotor retardation) not explained by the underlying medical condition. A recent review of case-finding instruments used in primary care showed that at least 11 questionnaires, ranging in length from 1 to 30 questions and ranging duration of administration from 1 to 5 min, have reasonable performance characteristics compared with a semi-structured interview that applies standard diagnostic criteria [25]. In busy oncology settings, clinicians commonly ask simple verbal questions about depressed mood or lack of interest in normal activities (anhedonia) to screen for depressed patients. A recent meta-analysis of this strategy showed that its positive predictive value was 57%, and its negative predictive value was 98%, thus making it more useful for excluding depression than for including it [21]. Patients, like Mrs. L, who screen positive for depressed mood should be more thoroughly evaluated. Risk factors for depression that should be evaluated in patients with pancreatic cancer include poor pain control, poor functional status, advanced disease, history of substance abuse, and poor social support. Recent losses and a personal or family history of depression are also risk factors. Major depression should be treated aggressively, even in patients with advanced pancreatic cancer, with supportive psychotherapy and judicious use of antidepressant medications.

Gastroparesis

Mr. C was a 62-year-old Hispanic man with the recent diagnosis of pancreatic cancer that involved the body of the pancreas, celiac, periportal lymph nodes and he also had a 2 cm metastatic lesion in the right lobe of the liver. His presenting symptoms were back pain, nausea, 20 pounds weight loss, and early satiety. His back pain was managed with sustained-release oral morphine and oral hydromorphone for "break-through" pain episodes. The patient and his oncologist decided in favor of systemic chemotherapy with weekly gemcitabine. Nausea became even more pronounced despite the use of prochlorperazine or ondansetron and was accompanied by vomiting and further weight loss. Chemotherapy was discontinued; however, there was no improvement in nausea or emesis. Patient presented to the emergency room with dehydration. An abdominal radiograph revealed a distended stomach, filled with fluid and food debris. An upper gastrointestinal contrast study was performed, which revealed no evidence of gastric or duodenal obstruction.

Gastroparesis is believed to affect over 60% of pancreatic cancer patients and is a "functional" form of gastric obstruction rather than a mechanical type of gastric outlet obstruction, which results from compression of the gastric pylorus and duodenum by a mass in the head of the pancreas. Cancer-associated gastroparesis may be accompanied by a generalized gastrointestinal dysmotility that results in ileus. Its consequences can be serious and include cachexia, dehydration, electrolyte imbalances, and impairment of quality of life. As in the case of Mr. C, this symptom complex can be exacerbated by chemotherapy-toxicity. The etiology of gastroparesis in most cases is thought to be antibody-mediated or secondary to neurological involvement by the malignant process. Comorbid conditions such as diabetes, hypothyroidism, and opioids contribute to worsening of symptoms of gastroparesis.

Diagnosis of Gastroparesis

The standard test for establishing diagnosis is gastric scintigraphy after a standardized solid meal of bread, jam, and egg substitute with 99 m Tc-sulfur colloid. Delayed gastric emptying is confirmed if more than 60% retention is present at 2 h or more than 10% is present at 4 h [26]. This investigation can be utilized both for diagnosis and monitoring of the effectiveness of prokinetic therapy [27]. A new, ambulatory method involves ingesting a radiotelemetry wireless capsule that measures luminal pH and pressure after being swallowed and transmits the data to a receiver worn by the patient (Smart Pill™, Buffalo, NY) [28]. Gastric emptying is detected by the sharp increase in pH as the capsule moves from the stomach to the duodenum.

Patients without weight loss, hypoalbuminemia, or other nutritional impairment can undergo dietary and behavioral modification, and be prescribed prokinetic and anti-emetic medications. However, for those with long-standing gastroparesis, correction of the fluid, and electrolyte disturbances, intravenous administration of

prokinetic and anti-emetic medications and nasogastric tube placement to decompress the stomach may be needed.

Dietary and Behavioral Modification

Dietary recommendations are based on measures that promote gastric emptying. Patient should be encouraged to sit erect, stand, or walk after a meal as gravity and body movement help in gastric emptying. Frequent small meals are encouraged, as is increasing the liquid nutrient component of meals, as liquids transit more rapidly than solids [29]. Lipids and indigestible fiber delay gastric emptying; thus a low-fat, low-residue, high carbohydrate meal is appropriate for patients with gastroparesis to avoid gastric distension and symptoms of bloating, satiety, and nausea.

Pharmacotherapy

Prokinetic agents and anti-emetic agents are the mainstay of therapy and the goals are to accelerate gastric emptying and prevent nausea or emesis. The commonly used prokinetic agents in the United States are metoclopramide and erythromycin. Tegaserod is no longer approved by the Food and Drug Administration (FDA). Cisapride and domperidone are not available in the United States but can be obtained under an investigational (IND) protocol.

Metoclopramide

Metoclopramide (Reglan®) is a 5-HT_4 receptor agonist and dopamine D_2 receptor antagonist. It promotes gastric emptying by facilitating gastrointestinal cholinergic and nitrergic (nitric oxide mediated) activity and improves gastric emptying and intestinal transit. Its anti-emetic properties are related to central and peripheral inhibition of dopamine receptors. At doses of 10–20 mg orally four times daily, metoclopramide results in subjective improvement in symptoms of nausea, vomiting, abdominal pain, postprandial fullness, nausea, and early satiety [30]. Metoclopramide readily crosses the blood-brain barrier, where D_2 receptor antagonism can cause akathisia or other extrapyramidal symptoms. Long-standing metoclopramide therapy, of over 3 months in duration, can result in irreversible tardive dyskinesia, in 1–10% of cases. Hence, this complication should be discussed before this medication is prescribed.

Erythromycin

Erythromycin is a bacteriostatic macrolide antibiotic with prokinetic properties that is widely used to treat diabetic gastroparesis. Erythromycin is a potent motilin

agonist that induces gastric peristalsis thus improving gastric emptying. Sturm et al. reviewed 36 clinical studies involving 514 patients who were treated with prokinetics for gastroparesis [31]. They concluded that erythromycin had the strongest effect on gastric emptying than did domperidone, cisapride, or metoclopramide. The side effects of high doses of erythromycin include abdominal pain, nausea, and vomiting secondary to increased gastrointestinal motility. Erythromycin also increased the risk of sudden cardiac death by as twice that of the control population [32].

Anti-Emetic Agents

Anti-emetic agents can be combined with prokinetic agents for a synergistic effect. Commonly used anti-emetics include phenothiazines such as promethazine, prochlorperazine or 5-HT_3 receptor antagonists such as odansetron and granisetron. In patients who do not experience response to one anti-emetic agent, another agent may be useful.

Botulinum Treatment

Small case series have reported improved symptoms and gastric emptying after the injection of botulinum toxin into the pylorus. This agent reduces the tone and phasic contractions of the pylorus by preventing cholinergic contractile activity [33]. The use of botulinum toxin for gastroparesis is considered off-label and prospective studies in patients with cancer-associated gastroparesis are lacking.

Surgical Management

Surgical intervention is increasingly used to treat refractory gastroparesis. The most common operation, gastric electrical stimulator implantation, has been performed in more than 1500 patients since 2001, mostly for diabetic gastroparesis. The gastric stimulator has been effective in the treatment of diabetic, idiopathic, and postsurgical gastroparesis. However, prospective studies of electrical stimulation in cancer-associated gastroparesis are needed.

Gastrostomy

Gastrostomy should be considered in refractory gastroparetic patients with severe nausea and vomiting. A gastrostomy tube can relieve symptoms, especially of interdigestive fullness, nausea, and bloating secondary to retained intragastric gas and liquids. Venting gastrostomy decreased symptoms, improved functional status

and weight in patients with idiopathic gastroparesis in a study with 3-year follow-up [34].

Jejunostomy

Cancer-associated gastroparesis patients with debilitating symptoms and nutritional compromise should be considered for jejunostomy tube placement. Before the placement of a permanent jejunal tube, a 48- to 72-h trial of nasojejunal feeding should be performed to confirm that the patient can tolerate the infusion of nutrients at a rate that delivers an adequate caloric and protein level [35]. Endoscopically placed jejunal tubes often migrate backwards into stomach, particularly in patients with recurrent vomiting. Thus, in patients requiring long-term enteral nutrition, surgically placed jejunostomy tubes are preferable to the endoscopically placed tubes. Enteral feeding with an iso-osmolar, nonelemental liquid supplement has been shown to be effective in the long-term care of patients with gastroparesis. Complications of the jejunal tube placement include infection, tube dysfunction, and tube dislodgment.

Parenteral Nutrition

Patients with cancer-associated gastroparesis may require parenteral nutrition if previous attempts of enteral nutrition have failed due to intolerance or enteral feeding complications. The morbidity of parenteral nutrition is considerable and includes vein thrombosis, sepsis, and hepatic cholestasis. Depending on a patient's prognosis and goals of care, parenteral nutrition should be considered after deliberation with patients and caregivers about risks and benefits. Periodic reassessment of the benefits of parenteral nutrition during the disease trajectory should be conducted, and if the risks are outweighed by benefits, it should be discontinued.

Jaundice

Ms. C was a 45-year-old Hispanic woman who presented with painless jaundice and 20 pounds of weight loss to her family practitioner. An ultrasound examination of the liver was recommended. This study revealed a mass in the head and uncinate process of the pancreas, dilatation of the common bile duct and pancreatic duct, and a solitary metastatic liver lesion. Laboratories revealed a serum bilirubin level of 7.8 mg dl − 1 and alkaline phosphatase level of 660 IU l − 1. She underwent an Endoscopic Retrograde Cholangio-Pancreatography (ERCP), which confirmed a stricture in the common bile duct. A plastic stent is placed across the common bile duct. After 2 weeks, the jaundice resolved. However, a month after stent placement, she developed recurrence of jaundice and fever.

The above case illustrates the typical presentation of a patient with cancer in the pancreatic head, with biliary obstruction resulting in jaundice. Palliation of jaundice in this instance can be achieved by surgical bypass (choledochojejunostomy) or nonsurgically by biliary stenting. Surgical therapy is considered for patients with resectable pancreatic cancer, or for patients with nonresectable, locally advanced but nonmetastatic cancer. In Ms. C′s case, the presence of liver metastasis was a clear indication for nonsurgical therapy. The potential advantages of surgical palliative therapy include the ability to add other procedures including celiac plexus block and gastrojejunal bypass for concurrent duodenal obstruction. The morbidity and mortality of these procedures is not minor, however, particularly in patients with inoperable pancreatic cancer. A recent retrospective review reported an overall mortality of 6% and mortality of 16% for unresectable pancreatic cancer patients who underwent palliative surgery [36]. The median survival for patients undergoing these procedures was 6 months only. Therefore, majority of these patients can be spared surgery and be palliated with nonsurgical stenting procedures.

Before endoscopic placement of biliary stents, ERCP evaluation is performed to evaluate the biliary tree and pancreatic duct. Preceding the procedure, antibiotics are administered prophylactically and coagulopathy corrected. The risk of stent occlusion increases after approximately 3 months. Elective stent exchange is reasonable if the physical condition of the patient is good. Endoscopic stent placement is safe and effective in this patient group; however, stents are prone to infection and occlusion from tumor ingrowth or debris [37]. Prophylactic administration of antibiotics and bile salts has not been shown to prevent stent occlusion. However, stent placement has lower morbidity and mortality as compared with biliary surgical bypass procedures.

Metallic stents are preferred to plastic, as these are wide-bored and less prone to occlusion and infection than plastic stents [38]. Metallic stents can be covered with a sheath (to prevent tumor ingrowth) or bare. The former type has a lower risk of occlusion but carry a significant risk of cholecystitis [39]. These are also easier to remove, in the case surgical resection is feasible. In all cases, if the patient has resectable disease, the surgeon should be consulted before the selection of the stent. As in Ms. C′s case, cholangitis is a common complication from biliary stents. Acute cholangitis is characterized by fever, jaundice, and abdominal pain that develops from biliary stasis and infection. Cholangitis can be a serious complication, and requires prompt intervention with antibiotics, intravenous fluids, and hospitalizations. In the majority of these cases, endoscopic stent replacement is required to reestablish biliary flow.

Cachexia

Ms. T was a 65-year-old Caucasian woman with a 6-month history of abdominal pain, backache, and asthenia. She experienced anorexia, abdominal bloating, flatulence, diarrhea, and 20-pound weight loss over the previous 6 months. Imaging studies revealed a pancreatic body mass with celiac adenopathy and encasement of

the superior mesenteric vein. Laboratory studies revealed an albumin of 2.8 g dl − 1 and fasting blood glucose level of 240 mg dl − 1. The patient received chemoradiotherapy and experienced a further 10-pound weight loss over the subsequent 2 months.

Ms. T's case vignette illustrates a commonly occurring problem in pancreatic cancer, "cancer cachexia." This condition is characterized by malnutrition, muscle wasting, weakness, and debility. When cachexia is associated with a failure of appetite responses, this condition is referred to as "anorexia-cachexia syndrome." Cancer cachexia occurs in >80% of patients with advanced pancreatic cancer. It can hasten death, reduce response to treatment, and exacerbate treatment toxicities [40]. The clinical stage of the malignancy (tumor burden) is not directly related to the extent of the cachexia. Indeed, small tumors in the pancreas can lead to significant weight loss, even in the absence of anorexia. Falconer et al. measured resting energy expenditure (REE) and found that patients had a higher REE than did control subjects [41]. Cancer patients have several metabolic abnormalities involving carbohydrates, amino acids, and lipids. Pancreatic cancer is associated with secondary diabetes in 50% of cases, and hyperglycemia alone is a negative prognostic factor in this disease [42]. The anorexia-cachexia process in advanced cancer appears to be mediated by circulating catabolic factors, either secreted by the tumor alone or in concert with host-derived factors, such as tumor necrosis factor-α (TNF-α), interleukins (IL) 1 and 6, gamma interferon (IFN-γ), and leukemia inhibitory factor [43].

Management of Cachexia

Medications such as glucocorticoids, megestrol acetate, and cannabinoids have the potential to stimulate appetite and increase weight but, unfortunately, have modest benefits for cancer cachexia.

Corticosteroids, such as dexamethasone, prednisolone, and methylprednisolone, result in short-term improvement in appetite, nausea, and energy. The usual dose of dexamethasone is 3–6 mg by mouth daily. Its exact mechanism of action is unknown; however, it is believed to interfere with inflammatory cytokines such as IL-1 and TNF-α [44]. Loprinzi et al. compared megestrol acetate, dexamethasone, and fluoxymesterone in a randomized control study of 475 patients with advanced cancer [45]. Fluoxymesterone resulted in the least improvement in appetite. Dexamethasone and megestrol showed a similar degree of benefit.

Megestrol acetate is a synthetic hormone that mimics progesterone in the body and interferes with hormone signaling. Megestrol improves appetite and quality of life in many patients but does not affect lean body mass or result in a change in performance status. It can cause weight gain, but body composition studies have indicated that megestrol increases body fluid and fat rather than lean body mass. Loprinzi et al. [46] randomly assigned 342 patients with cancer cachexia to receive megestrol acetate dose of 160, 480, 800, or 1280 mg day^{-1}. Patients who received 800 mg day^{-1} reported the greatest improvement in appetite and food intake. Fifteen

percent of patients treated with 800 mg of megestrol experienced weight gain; lower weight gains were noted in the other dose groups. There was also a trend toward higher serum albumin levels in the 800 mg day^{-1} group. Deep-vein thrombosis, hyperglycemia, adrenal insufficiency, and androgen deficiency in male patients are important adverse effects of megestrol therapy. Deep-vein thrombosis occurs in 2–18% of patients and hyperglycemia in 2–13% of patients receiving megestrol and is an important consideration in pancreatic cancer patients, who are predisposed to coagulopathic events and may have underlying diabetes.

Cannabinoids are marijuana derivatives; they act by interacting with cytokines or with endocannabinoid receptors in the brain limbic system and hypothalamus or in the peripheral organ systems. They work in palliating cachexia in cancer patients by stimulating appetite. Cannabinoids have anti-emetic properties and elevate mood. Their adverse effects include dysphoria, confusion, dizziness, loss of coordination, fluid retention, vomiting, and impotence [47]. The superiority of cannabinoids to steroids remains to be proven. Jatoi et al. [48] compared 2.5 mg dronabinol twice daily, 800 mg megestrol daily, and the combination in 469 advanced cancer patients. Patients reported greater appetite improvement and weight gain with megestrol. The combination of both drugs did not result in additional benefit.

Ten percent of patients with pancreatic cancer have a new onset of diabetes [49]. Cachectic cancer patients have glucose intolerance, which can contribute to weight loss as illustrated in the above vignette. Control of hyperglycemia in this case can improve cachexia symptoms. Lundholm et al. [50] randomized 138 patients with mainly advanced gastrointestinal malignancies to receive insulin plus best palliative support or best palliative support alone. They found that although overall daily caloric intake did not change between the groups, carbohydrate intake was significantly increased by insulin. Adequate control of hyperglycemia has been associated with improved survival in cancer patients.

Currently, treatments under investigation for cancer cachexia include omega-3 fatty acids (eicosapentaenoic acid and docosahexaenoic acid), amino acids including L-carnitine, nonsteroidal anti-inflammatory drugs, thalidomide, and ghrelin mimetics.

Nutritional Management

The goals of nutritional care are to improve caloric and nutrient intake, body composition, functional or performance status, immune function, and quality of life. Oral or enteral nutritional supplements are beneficial for patients whose quality of life and survival may be enhanced by anticancer therapy. Enteral nutrition is preferred over parenteral methods for patients with a functioning gastrointestinal tract [51]. Gastric enteral feedings are sufficient for most patients. However, patients at a high risk for aspiration, gastroparesis, gastric outlet obstruction, or who have a history of previous gastric surgery typically require jejunostomy feedings. Ideally, a registered dietitian (RD) should intervene at the initial diagnosis of cancer. Regular, consistent contact via telephone or face to face is encouraged. The role of the RD

should include the following: calculating nutrient and fluid consumption, evaluating nutritional status using patient recall data, anthropometrics, and laboratory indices, anticipating the nutritional risks of both the cancer and its treatment and managing nutrition-related adverse effects of cancer therapy. Encouraging patients to take a nutritional supplement at fixed times throughout the day (as when taking medication) can help optimize nutritional intake. Patients should aim to ingest a supplemental 300–600 Kcal daily in addition to regular meals [52]. Bauer et al. [53] evaluated compliance with nutritional prescriptions and their effect on outcomes in patients with unresectable pancreatic cancer. They found that compliance with a prescription of 1.5 cans of a protein- and energy-dense, oral nutrition supplement (with or without eicosapentanoic acid) improved total dietary intake and body weight. They also found that this level of supplement intake did not inhibit meal intake.

Pancreatic enzymes: Approximately 65% of patients will have some degree of fat malabsorption, and 50% have protein malabsorption [54]. Ms. T experienced the symptoms of fat malabsorption, including diarrhea, steatorrhea, flatulence, abdominal pain, and bloating. Pancreatic enzyme replacement can be used to treat nutrient malabsorption caused by pancreatic insufficiency. Enzyme replacement has been shown to improve the above symptoms and prevent further weight loss [55]. The goal of enzyme replacement therapy is to achieve normal enzyme activity in the duodenum. Current clinical practice involves the administration of 25,000–40,000 units of lipase/meal using pH-sensitive pancrelipase microspheres, along with dosage increases, compliance checks, and differential diagnosis in cases of treatment failure [56]. Ideally, patients should ingest enzymes with the first bite of every meal and snack and midway through the meal, to maximize enzymatic activity in the duodenal lumen simultaneously with the meal. Patients should maintain a detailed food and bowel diary to determine which foods and enzyme dosage are best tolerated.

Constipation

Mr. K was a 72-year-old African American man with pancreatic adenocarcinoma, involving the body of the pancreas with celiac adenopathy. He had considerable abdominal and back pain and was prescribed oral short-acting opioid analgesic and sustained-release oral morphine formulation. He was advised prophylactic use of a stool-softener to prevent constipation. Patient presented to the clinic 1 week later, complaining of bloating, constipation, nausea, and abdominal distention. He was advised a combination of a stimulant laxative and stool softener along with liberal oral hydration. No improvement results after 48 h of therapy. An osmotic laxative was added; laxation resulted and symptomatic relief occurred.

Constipation is a frequently occurring problem in pancreatic cancer patients and affects over 50% with an advanced disease stage. The distress resulting from this symptom equals that with cancer pain. The etiology of constipation in pancreatic cancer patients is multifactorial. Constipation may result from direct tumor invasion

into the transverse colon or into the enteric nerves and muscles, as a part of a larger paraneoplastic phenomenon, which may be hormonal or cytokine-mediated and accompanied by generalized gastrointestinal dysmotility including gastroparesis, or from the effects of treatment such as opioid analgesics and 5-HT_3 antagonists [57]. Over 60% of cancer patients require laxatives even without opioid usage while this figure approaches 90% with the use of concurrent opioids. The functional effect on the opioid receptors is to reduce peristalsis and increase intestinal circular muscle resting tone. In addition, opioids also alter intestinal fluid handling as a result of decreased transit time in the small bowel and decreased secretory gut function. The advanced age, poor appetite resulting in suboptimal intake of dietary fiber and fluids as well as poor performance status of pancreatic cancer patients contribute to the problem of constipation [58].

The current strategies for management include early intervention with patient education, dietary counseling, and agents to induce laxation. Mumford estimated that an increase of dietary fiber intake by 450% would be required so as to increase laxation by 50% in cancer patients receiving radiotherapy and suffering from constipation [59]. This degree of increased dietary fiber intake is not feasible in this patient group. The fluid requirement is also considerable: 1.5–2 l of oral fluid intake daily is needed to have a positive effect. Only a weak correlation exists between the dose of the narcotic used and the degree of constipation. Transdermal fentanyl may be less constipating than morphine or hydromorphone [60]. Methadone is also associated with a lower laxative requirement than morphine [61]. In the case of Mr. K, both the narcotic agents that were prescribed have constipation as a side-effect.

Commonly used pharmacological interventions can be grouped into the following categories: stool softeners, which act as detergents that enhance dispersion of fluid into the stool content; osmotic agents such as polyethylene glycol, lactulose, and sorbitol that withdraw fluid into the intestinal contents; and bulk fiber, such as psyllium which requires adequate oral hydration to be effective. Most patients receiving narcotics should be prophylactically started on a regimen of stool softener + stimulant laxative. This combination was also prescribed for Mr. K, but the results were suboptimal. In resistant cases, an osmotic agent is added. This was also the case with Mr. K, where the addition of lactulose to the combination of stimulant laxative and stool softener was needed. This regimen needs close monitoring so that changes can be instituted in dosage as needed and fecal impaction avoided.

The FDA has recently approved methylnaltrexone, a peripherally acting mu-opioid receptor antagonist, which selectively reverse opioid actions mediated by receptors outside the central nervous system, while preserving centrally mediated analgesia. Methylnatrexone is subcutaneously administered and was investigated in two randomized, double-blind placebo-controlled studies involving a total of 287 patients who were suffering from opioid-induced constipation that was not relieved with laxatives usage [62, 63]. In both studies, all patients had advanced late-stage illnesses with a life expectancy of less than 6 months. Prior to treatment with methylnaltrexone, patients had either less than three bowel movements in the week prior to treatment or no bowel movement for more than 2 days. Patients

receiving methylnaltrexone achieved a significantly higher rate of laxation within 4 h of dosing versus placebo (62% and 58% vs. placebo, 14%; $P < 0.0001$ for both). This agent has to be administered subcutaneously and its side-effects include gastrointestinal perforation, abdominal cramps, flatulence, diarrhea, nausea, and dizziness and is contraindicated in patients with advanced illness with impaired gastrointestinal wall integrity. Naldemedine, an oral, mu-opioid receptor antagonist, also has been approved for opioid-induced constipation in noncancer pain and may be considered for off-label use in cancer patients. Oral naloxone and naloxegol, a pegylated form of naloxone, appears to be effective for opioid-induced constipation in the noncancer patient population but concern for reversal of analgesic effect exists.

Malignant Ascites

Ms. D was a 45-year-old African American woman with metastatic pancreatic cancer that involved the liver, retroperitoneal nodes and omentum. Patient was treated with systemic gemcitabine followed by capecitabine chemotherapy; disease progression resulted and further chemotherapy was discontinued. She experienced abdominal distention and lower extremity edema, and the patient was treated with diuretics. No improvement in distention resulted. She then experienced exertional dyspnea and abdominal pain from progressive ascites. A large-volume (3 l) ascitic paracentesis was performed; a cytologic examination revealed adenocarcinoma. The fluid albumin level was 1.5 g dl − 1 (serum albumin was 2.2 g dl − 1). She experienced relief for 2 weeks, which was followed by reaccumulation of peritoneal fluid.

Malignant ascites results either from direct peritoneal invasion by the cancer or secondary effects of the underlying malignancy, such as venacaval or portal obstruction, lymphatic blockade, hypoalbuminemia, or enhanced vascular permeability secondary to cytokine release (including TNF, vascular endothelial growth factor, IL-6, and vascular permeability factor) [64]. The treatment of malignant ascites differs from that of ascites associated with hepatic cirrhosis, which is the most common cause of ascites in adults and therefore the management options differ. Ms. D experienced direct peritoneal invasion and therefore she had positive cytology. However, malignant cytologic characteristics occur in 50–60% of the cases and the overall sensitivity of cytological analysis in this condition is <75% [65]. An examination of ascitic fluid can provide clues as to the underlying etiology. Even in the absence of positive cytologic findings, low levels of ascitic glucose and a low serum-ascites albumin gradient (serum albumin-ascitic albumin <1.1 g dl^{-1}) are suggestive of carcinomatosis as in the case of Ms. D. An elevated serum-ascites albumin gradient, on the other hand, suggests portal hypertension or lymphatic blockade from the tumor [66].

Malignant ascites may resolve after the underlying cancer is treated with antineoplastic therapy. The goal of therapy is palliative so as to relieve ascites-induced discomfort and improve quality of life. Ms. D experienced no symptom

improvement from diuretic therapy, but the use of diuretics for malignant ascites is controversial. Lee et al. reported that diuretics were used by 61% of physicians to treat malignant ascites but by only 45% noted a benefit [67]. No randomized controlled trials have assessed the effectiveness of diuretic therapy in the treatment of malignant ascites. In the prospective study by Pockros and colleagues, a response to diuretics occurred in patients with liver metastases and a serum-ascites albumin gradient >1.1 g dl^{-1}, whereas patients with ascites caused by peritoneal carcinomatosis or chylous malignant ascites who had no portal hypertension and a serum-ascites albumin gradient <1.1 g dl^{-1} did not experience any benefit from the diuretics [68]. These data suggest that serum-ascites albumin gradient may serve as a useful guide for predicting response to diuretics.

Therapeutic paracentesis is the only available option for providing rapid symptom relief from malignant ascites. The ideal rate of fluid withdrawal has not yet been determined, but large-volume paracentesis, of up to 5 l, is usually safe. McNamara et al. performed a prospective study to determine how much fluid needs to be drained for symptom relief [69]. A significant improvement in abdominal pressure was found with the removal of a median of 4.9 l (range = 0.8–15 l). The complications of paracentesis include hypotension, renal failure, peritonitis, hypoalbuminemia, and pulmonary embolism. In ascites secondary to cirrhosis, concurrent albumin or plasma expanders have been shown to prevent circulatory collapse. However, there are no data to support their use in patients with malignant ascites. Rosenberg et al. performed a retrospective analysis of patients undergoing therapeutic paracentesis for malignant ascites; a median of 6 paracentesis was performed per patient [70]. The median interval between procedures was 10 days. Indwelling tunneled or nontunneled catheters can be considered for patients requiring frequent paracenteses. Tunneled catheters have a low risk of infection (2.5% in one retrospective series). Nontunneled (pigtail catheters) have a higher infection risk (as high as 30%) and are not recommended other than for patients with terminal disease.

Peritoneo-venous shunts are a one-way valve containing systems that direct peritoneal fluid to the vena cava while preventing reflux. These shunts (Leveen or Denver) are widely used in cirrhotic patients with ascites and can provide palliation for malignant ascites in refractory cases. Their complications include occlusion, disseminated coagulation, and a theoretical risk of tumor dissemination. Breast and ovarian cancer patients can be benefited from this surgical procedure. However, gastrointestinal cancer patients, particularly those with pancreatic cancer, are not appropriate candidates for this surgery, due to the poor prognosis [71].

Vascular Thrombosis

Mr. P was a 72-year-old Caucasian male with the diagnosis of locally advanced, unresectable cancer of the pancreatic body and with periportal adenopathy. He underwent systemic chemotherapy with gemcitabine. After 3 months of chemotherapy, he underwent a computed tomography scan, for tumor restaging. A minimal increase in the tumor mass was noted along with a new portal vein thrombus. Serum

CA 19–9 level increased from 340 IU ml − 1 before treatment to 1068 IU ml − 1 after treatment. Computed tomography scan of the chest reveals a pulmonary embolus in a distal branch of the right pulmonary artery. Doppler sonography of his lower extremities reveals right popliteal venous thrombus.

Pancreatic cancer is associated with a high risk of thromboembolic disease and which is related to an intrinsic hypercoagulable state. Tissue factor, an important procoagulant, is expressed by tumor cells and activates the extrinsic coagulation pathway [72]. Tissue factor also upregulates the vascular endothelial growth factor (VEGF) and downregulates thrombospondin leading to an angiogenic phenotype. Expression of tissue factor has been associated with an adverse outcome in pancreatic cancer. Other factors inducing thrombosis are thrombin and circulating carcinoma mucins, including CA 19–9, which activate thrombosis via platelet aggregation. Mutated k-ras on the other hand activates thrombosis by decreasing thrombospondin concentration; k-ras is also associated with increased angiogenesis. Other factors that contribute to hypercoagulability in pancreatic cancer include the use of cytoxic chemotherapy, surgical procedures, hospitalization, venous stasis from restricted mobility, and vascular obstruction from lymphadenopathy (as in the case of Mr. P) and metastatic liver disease.

The incidence of thromboembolic disease in pancreatic cancer is higher than in other metastatic cancers and ranges from 12% in clinical and 47% in autopsy series [73]. In a prospective trial of gemcitabine + erlotinib versus gemcitabine + placebo, Moore et al. reported a 14% incidence of vascular events. In this and other studies, thromboembolic disease correlated with an adverse clinical outcome [74]. Lower extremity venous thrombosis, thrombophlebitis migrans, portal vein thrombosis, and pulmonary thromboembolism are the common manifestations of thromboembolic disease in pancreatic cancer. Other manifestations include disseminated intravascular coagulation, splenic vein thrombosis, mesenteric vascular thrombosis, and venous gangrene or extremity ischemia.

At the current time, patients with thromboembolic disease due to pancreatic cancer should be considered for anticoagulation with low-molecular weight heparin or unfractionated heparin followed by long-term oral coumarin anticoagulant therapy. Mr. P had portal vein thrombosis, which has unique clinical features. In his case, there were no associated complications, such as portal hypertension, ascites, varices, and pain. In patients with these complications, portal vein stenting can be considered. However, the stent reocclusion rate is high, and the survival benefit of this approach is unknown. Thrombolytic therapies have been used particularly for acute thrombotic events, but the complication rate is high, and therefore, it cannot be recommended at this time. Portal vein thrombosis, particularly of recent onset, can be safely treated with anticoagulation therapy. All patients with pancreatic cancer, who undergo surgery, should be considered for prophylactic anticoagulation.

Low-molecular weight heparin has been proven to be superior to coumarin in prospective studies. Administration of the latter is complicated in patients with metastatic pancreatic cancer because of gastrointestinal symptoms such as emesis, concurrent chemotherapy, antibiotics, liver dysfunction, and malnutrition that results in vitamin K deficiency. The randomized comparison of low-molecular weight

heparin and oral anticoagulant (CLOT) study reported a 17% thromboembolic risk with coumarin as compared with a 9% risk with dalteparin [75]. Only 46% of the patients randomized to the coumarin arm had therapeutic anticoagulation in this study despite intensive monitoring. Therefore, low molecular weight heparin is preferred to coumarin anticoagulation for the treatment of thromboembolic disease in cancer patients. In the CLOT study, the investigators also reported a significantly higher mortality with coumarin, as compared with the low-molecular weight heparin arm (20% vs. 35% at 12 months, $p = 0.03$). These data have raised the discussion regarding prophylactic anticoagulation for pancreatic cancer patients, to favorably impact both thrombosis and early mortality.

As per National Comprehensive Cancer Network (NCCN) guidelines for the treatment of venous thromboembolism in cancer patients [76]: (1) all hospitalized cancer patients should be considered for thromboembolic prophylaxis with anticoagulants in the absence of bleeding or other contraindications; (2) routine prophylactic anticoagulation is not recommended, with the exception of patients receiving thalidomide or lenalidomide; (3) patients undergoing major surgery for malignant disease should be considered for pharmacologic thromboprophylaxis; (4) low molecular weight heparin is the preferred agent for both the initial and continuing treatment of cancer patients with established VTE; and (5) the effect of anticoagulants on cancer patient survival requires additional study and cannot be recommended at present.

Three low-molecular weight heparins have been approved for clinical usage in the United States: enoxaparin, dalteparin, and tinzaparin. Fondaparinux has also been approved, but is a pentasaccharide and not a heparin. There are no known differences between these, in terms of effectiveness, and there are few comparative studies. Wells et al. compared initial therapy of either tinzaparin or dalteparin followed by coumarin in thromboembolic disease and reported no significant differences between the treatment arms in either recurrent thrombotic or bleeding events [77]. They concluded that tinzaparin and dalteparin were safe and effective in outpatient treatment.

The direct oral anticoagulants, including the direct factor IIa inhibitor dabigatran and the factor Xa inhibitors apixaban, rivaroxaban, and edoxaban are under investigation in cancer patients. These agents offer practical benefits over traditional anticoagulants including ease of administration without frequent laboratory testing for monitoring coagulation parameters and reduced food interactions. However, data regarding long-term safety and efficacy in cancer patients is lacking. Subgroup analysis of the recent EINSTEIN-DVT study did investigate the safety and efficacy of rivaroxaban in patients with active malignancy and demonstrated no significant difference in venous thromboembolic recurrence or bleeding complications between the rivaroxaban and low molecular weight heparin followed by warfarin [78]. However, increasingly low molecular weight heparin is being used long-term instead of warfarin and this trial did not use the latter in the control arm. Given the limited prospective clinical trial data demonstrating the safety and efficacy of direct anticoagulants in cancer patients and lack of appropriate control arms, current published guidelines do not recommend their routine use in patients with cancer.

The use of inferior vena cava (IVC) filters in patients should be only in patients with contraindications to anticoagulation or in case of anticoagulation failure [79]. IVC filters offer short-term protection from pulmonary embolism but are associated with higher rates of deep vein and filter-site thrombosis compared with no filters and have not shown to offer any survival benefit.

Conclusion

- Treatment goals should be reviewed with the patient from the outset. Patients who are educated regarding their prognosis, treatment alternatives, and likelihood of success or failure can make informed treatment choices.
- Depressive symptoms are common in pancreatic cancer and often associated with uncontrolled pain, poor functional status, advanced disease, and inadequate social support. The diagnosis follows standard definitions and treatment is not substantially different from the primary-care setting.
- Gastroparesis is an under-recognized and therefore undertreated problem, despite its common occurrence. Early satiety, nausea, and cachexia in the absence of mechanical gastric outlet obstruction should raise suspicion. Nuclear gastric emptying study is useful to establish the diagnosis. Dietary and behavioral modification, prokinetics, and anti-emetics form the cornerstone of therapy. In refractory cases, enteral tubes and nutritional supplementation are required.
- Cachexia in pancreatic cancer is multifactorial in etiology and unrelated to the stage of the disease. Steroids and anti-inflammatory agents are used in its pharmacotherapy. Control of hyperglycemia, nutritional support, and pancreatic enzyme supplementation improves symptoms and prevents further weight loss.
- Ascites resulting from pancreatic cancer can be secondary to the tumor or its effects such as vascular or lymphatic blockade. Low serum to ascites albumin gradient is suggestive of carcinomatosis. Large-volume paracentesis is safe, and in case of recurrent ascites tunneled indwelling catheters are preferred. Non-tunneled pigtail catheters are associated with a higher infection risk.
- Constipation results not only from the use of opioid analgesics, but as a result of paraneoplastic and tumoral infiltration. Stimulant laxatives and stool softeners are the preferred initial approach, along with oral hydration. In resistant cases, osmotic laxatives are needed. The advent of peripheral opioid receptor antagonists, such as methylnaltrexone, has introduced a new paradigm in the management of this condition.

Cross-References

▶ Clinical Decision-Making in Pancreatic Cancer
▶ Palliative Surgery in Advanced Pancreatic Cancer
▶ Paraneoplastic Syndromes in Pancreatic Cancer

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Therapeutic Endoscopy in the Management of Pancreatic Cancer

Alyson McGhan and Rebecca Burbridge

Contents

Abstract

Endoscopic retrograde cholangiopancreatography (ERCP) is a safe and reliable method for the diagnosis, treatment, and palliation of pancreaticobiliary malignancy. In 2015, the estimated new cases of pancreatic cancer were about 48,960. The 5-year survival of pancreatic cancer is dismal at approximately 5% Siegel et al. (CA: Cancer J Clin 65(1):5–29, 2015). ERCP provides less invasive approaches to diagnosis with examination of the biliary and pancreatic ducts. The procedure also allows for therapeutic relief of biliary obstruction. Advanced endoscopic techniques offer palliation of symptoms related to advanced

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pancreatic cancer and improvement in quality of life. This chapter addresses the use of therapeutic endoscopy in the diagnosis and management of pancreatic adenocarcinoma.

Keywords
ERCP · FISH · Cholangioscopy · Intraductal stricture biopsy · Self-expandable metal stent · Fiducial · Celiac plexus neurolysis

Overview of ERCP

Since its inception in 1968, endoscopic retrograde cholangiopancreatography (ERCP) has become widely used in a variety of pancreaticobiliary disorders. Over the decades, the therapeutic and diagnostic applications of ERCP have changed to match the improvement in its technology for direct and nondirect visualization in conjunction with improvement in therapeutic devices and techniques.

ERCP combines endoscopy with fluoroscopy. Usually sedated by monitored anesthesia care or general anesthesia, a side-viewing endoscope, or duodenoscope, is passed through the patient's mouth and into the duodenum in order to visualize the ampulla of Vater. Cannulation of the desired duct is required for successful diagnostic and therapeutic ERCP. Cannulation is done with the use of sphincterotomes and guidewires in order to gain access. Once access is achieved, ductal anatomy is defined by injection of contrast into the biliary or pancreatic ducts, while fluoroscopy is employed for visualization. When the desired duct is accessed, several devices can be deployed such as biopsy forceps, stents, or balloon dilators for the goal of diagnosis or management of multiple pancreaticobiliary disorders.

Though ERCP is relatively safe, even in a skilled endoscopist's hands, the overall rate of adverse events approaches approximately 5–10%. Consensus definitions of adverse events related to endoscopic sphincterotomy and its severity grading were first introduced in 1991 and still widely used today. The most common adverse complication is pancreatitis which occurs in about 5% of cases. Post-ERCP pancreatitis can often require hospitalization for treatment and can be severe, requiring prolonged hospitalization and need for intensive care. Other common adverse events include bleeding, infection, and perforation. Death is rare and occurs in $<0.5\%$ of cases and is typically related to cardiopulmonary events related to sedation.

Diagnosis

Most patients with pancreatic adenocarcinoma present with obstructive jaundice, which unfortunately is a sign of advanced disease. Suspected pancreatic malignancy is often evaluated with multiple imaging modalities including CT or MRI; however, the diagnosis of pancreatic cancer relies heavily on the identification of a pancreatic

mass. Unfortunately, there are many instances in which there is no identifiable mass and ERCP is then necessary. The role of ERCP in the evaluation of pancreatic malignancy includes visualization of the ductal anatomy as well as histologic or cytologic tissue sampling. Endoscopic ultrasound has largely overtaken ERCP for the diagnosis of pancreatic head adenocarcinomas. However, ERCP with brush cytology and biopsy may be required for the evaluation of pancreatic duct strictures or in cases with nondiagnostic EUS sampling [1].

Endoscopic Features

Ductal adenocarcinoma is the most frequent type of pancreatic adenocarcinoma. It has nonspecific early symptoms, and there are no general tests for screening. ERCP findings suggestive of pancreatic cancer include stricture of both the bile and pancreatic ducts with upstream dilation, also known as the double duct sign. In a single center retrospective study, 355 patients with pancreatic duct strictures on ERCP were reviewed. The study revealed that 65% of patients with a double duct sign were diagnosed with pancreatic cancer resulting in a sensitivity of 76.7% and positive predictive value of 65%. Another endoscopic feature that was strongly associated with malignancy was stricture location within the pancreatic head or neck (odds ratio of 42, $p < 0.0001$) [2]. Overall, ERCP reaches a sensitivity of 80–90% for the diagnosis of ductal pancreatic adenocarcinoma.

Stricture features may assist in predicting prognosis of pancreatic cancer. Length of the stricture has been predictive of tumor size and stage. In a small retrospective study of 18 patients who underwent ERCP prior to surgery between 1991 and 1996, stricture length of biliary and pancreatic strictures was measured and compared to surgical specimens [3]. Pancreatic duct stricture length measured on ERCP correlated with both size (<0.001) and stage (<0.002) in resectable pancreatic cancer [4, 5].

Tissue Sampling

A key role in diagnostic ERCP is for tissue sampling via brushing, biopsy, or aspiration in order to achieve a definitive diagnosis. While tissue sampling should have a high sensitivity and specificity, simple to perform, and reliable, this is not always the case for pancreaticobiliary malignancy requiring diagnostic ERCP. Thus, diagnosis remains dependent on the technical skill of the endoscopist and the resources available within that center.

Malignant strictures of the bile duct are commonly caused by pancreatic cancer, cholangiocarcinoma, or periampullary carcinomas. Malignant strictures are difficult to differentiate from benign strictures caused by bile duct stones or inflammatory strictures due to chronic pancreatitis. The incidence of benign disease on pathology after pancreaticoduodenectomy for a presumed malignancy is estimated at 5–13% [6]. Confirming malignancy through tissue diagnosis is important before considering more aggressive surgical or medical management. The techniques utilized for tissue

sampling during ERCP remains important for adequate diagnosis of pancreatic cancer.

Brush Cytology

Brush cytology was first introduced in the 1970s for the diagnosis of malignant biliary or pancreatic strictures. Even now, brush cytology performed at ERCP has become the preferred initial method for tissue sampling of pancreaticobiliary ductal strictures. Several endoscopic factors can influence the yield of cytology including length of stricture, tightness of stricture, and location of the stricture. The quality of cytological specimen also affects the diagnostic yield including processing technique, cellularity, cellular preservation, and quantity of diagnostic cells. Though brush cytology is commonly utilized, the sensitivity has been reported to be as low as 30% [7]. Not only is the collection of biliary duct cytology important in diagnosis but the interpretation by an expert cytopathologist is paramount. In a study by Wight and colleagues, 129 biliary brushings from 120 patients were reviewed. The sensitivity of diagnosis of malignancy was increased from 49.4% to 89% when two expert cytopathologists reviewed the specimens instead of one cytopathologist. The authors deduced that this difference was due to the increased accuracy in diagnosis when more time was taken to review the specimen and the second reviewer reclassified "atypical" or "suspicious" results to malignancy [8]. Despite the ease of obtaining brush cytology, low complication rate, and low cost, the sensitivity of routine cytologic analysis remains suboptimal. Multiple studies have reported a specificity of nearly 100%; however, there is a wide range of sensitivity from 30% to 80%. There have been various attempts at increasing the yield of brush cytology such as dilating the stricture prior to brushing, new brushing devices, and use of supplemental testing and biomarkers.

Increased knowledge of cancer biology and genetics has paved the way for improved detection of malignancy. The use of fluorescence in situ hybridization (FISH) has advanced the yield of brush cytological sampling. Malignant cells typically have chromosomal abnormalities such as aneuploidy and gene deletions. These chromosomal alterations are visible with the use of FISH whereby fluorescently labeled DNA probes are used to identify specific chromosomal loci. In a study of 131 patients who were being evaluated for malignant biliary strictures, the sensitivity of cytology and FISH for the detection of bile duct brushing specimens were 15% and 34% ($p < 0.01$), respectively. The specificity of cytology brushings and FISH were 98% and 91% (p-0.06), respectively [9]. This study suggests that FISH can be used as an adjunct to routine brush cytology for the evaluation in patients suspected of having a malignant bile duct stricture. Gonda et al. [10] assessed 76 patients with indeterminate biliary strictures and revealed polysomy FISH had increased the sensitivity of brush cytology from 21% to 58% with a specificity of 98%. With the additional evaluation of institutional cost per patient of FISH, Gonda et al. concluded that the use of FISH should be limited to the evaluation of strictures with previously nondiagnostic cytology examination.

Brush cytology alone is not sufficient for the diagnosis of malignant biliary strictures, therefore alternative and supplemental methods for tissue sampling and cytohistopathologic examination is necessary.

Stricture Biopsy

Due to the low sensitivity of brush cytology, other methods for tissue collection have been developed. Biopsy of bile duct strictures with forceps was introduced in the early 1990s.

Stricture biopsy provides information about tissue structure as well as details on tissue invasion depending on the depth of the biopsy, unlike brush cytology. The data has been mixed about whether stricture biopsy has significantly improved clinical utility compared to brush cytology. In a recent review of 241 patients with biliary strictures at a single institution who underwent transpapillary brush cytology or forceps biopsy, the investigators looked to evaluate the diagnostic yield of brush cytology and biopsy forceps. The study revealed that the sensitivity of forceps biopsy for malignant biliary strictures was higher than that of brush cytology (60.6% vs. 36.1%, $p < 0.01$) [11]. In a meta-analysis of nine studies, the pooled sensitivity of intraductal biopsy and brush cytology in diagnosing malignant biliary strictures were 48.1% and 45%, respectively. A combination of both only modestly increased sensitivity to 59.4% [12]. Based on this meta-analysis, the use of both brush cytology and intraductal stricture biopsy during ERCP increases the yield in the diagnosis of malignant biliary strictures; however, the combined approach still has a relatively low diagnostic yield for malignancy.

Ductal biopsies may be more difficult to obtain as forceps are not passed over a wire and may pose a greater risk of perforation when the forceps are advanced through a fresh sphincterotomy. Unlike biliary brushings, biopsies are not obtained as often, may be difficult to obtain because of fibrosis, require greater technical skill, involve more time, and pose a slightly increased risk. There are differences of opinion on the value of biopsies, however. The use of a combined (i.e., brushing plus biopsy) approach may be employed in indeterminate strictures in whom there is a high suspicion for pancreatic cancer.

Fluid Aspiration and Molecular Analysis

Prior to the widespread use of EUS-FNA, cytologic examination of pancreatic juice aspirated during ERCP was utilized; however, the sensitivity of pancreatic juice aspiration ranges from 33% to 76%. Several tumor markers are tested to aid in the diagnosis of pancreatic cancer, including K-ras, CA 19-9, p53, and Span-1. Of the tumor markers, K-ras has been the most studied. Mutation of K-ras oncogene is one of the most common gene alterations in human malignancies and frequently found in pancreatic adenocarcinoma, occurring in up to 76% of cases. Multiple analyses have demonstrated that K-ras is a valuable molecular marker and independent diagnostic

tool for pancreatic cancer. K-ras in pancreatic juice has a higher sensitivity and specificity than that of serum K-ras; however, the biggest drawbacks of pancreatic juice K-ras include the high false-positive rate [7, 13, 14]. K-ras can be found in up to 25% of cases of chronic pancreatitis without evidence of malignancy. These results have been supported by other studies which have noted detectable K-ras in chronic pancreatitis or even in normal pancreas without evidence of malignancy on follow-up [15]. Serum carbohydrate antigen, CA 19-9, and Span-1 have also been associated with the presence of malignant pancreatic cells collected in bile duct aspiration, and CA 19-9 levels may indicate response to chemotherapy for pancreatic cancer; however, there is no consensus on the routine use of these tests on aspirates [16].

Cholangiopancreatoscopy

Over the recent years, much more focus has been placed on direct visualization of the biliary and pancreatic ducts. High-definition visualization with cholangiopancreatoscopy enhances the diagnostic yield of ERCP, particularly in patients with indeterminate biliary strictures. The advent of single operator cholangioscopy has improved the sensitivity and specificity in the diagnosis of malignant biliary strictures. Miniature endoscopes and optical catheters are passed through the working channel of a therapeutic duodenoscope during ERCP. Fiberoptic cholangioscopes range in 3.1–3.4 mm in diameter with a working channel of 1.2 mm that allow for biopsy forceps to be passed. Cholangioscopic findings suggestive of malignancy include the presence of easy oozing, irregular surface, as well as the presence of irregular, dilated, tortuous vessels called tumor vessels [17]. Though the tumor vessel may predict malignancy, it still does not help differentiate between biliary or pancreatic malignancy. In a prospective multicenter trial of cholangioscopy, a subgroup analysis of 95 patients demonstrated a sensitivity of the diagnosis of malignancy of 51% with ERCP impression, 78% with cholangioscopy impression, and 49% with cholangioscopy-directed biopsy. Specificity of each modality was 54%, 82%, and 98%, respectively [18]. Cholangioscopy has been used as an adjunct to ERCP in patients with previously diagnosed indeterminate strictures. Among 18 patients with indeterminate strictures who underwent cholangioscopy, 11 (61%) had a final diagnosis of malignancy [19]. In another recent large prospective multicenter study out of Japan, a total of 148 patients were enrolled for the diagnosis of indeterminate biliary or pancreatic strictures and for treatment of pancreaticobiliary disease. The procedure success rates for identifying the target lesions were 91.2%. Adequate tissue for histologic exam was secured in 81.4% of all patients who underwent directed biopsy by cholangiopancreatoscopy. Specifically, in those with pancreatic duct lesions, adequate tissue by direct biopsy was secured in 90.9% of patients. The incidence of adverse events was 5.4% [20]. Complications specific to cholangiopancreatoscopy include higher rates of cholangitis and pancreatitis related to intraductal irrigation compared to standard ERCP. As biliary endoscopists have become more familiar with cholangioscopy, there have been advances in the

modalities of visualization. Several techniques have included narrow band imaging (NBI), confocal endomicroscopy, as well as intraductal ultrasonography.

Cholangioscopy in combination with standard ERCP and brushings has increased the sensitivity for the diagnosis of malignant biliary strictures. Though the use of cholangioscopy has increased, it is still limited to centers with advanced biliary endoscopists and remains a technically challenging procedure.

Therapy

Surgery is the curative treatment of choice for pancreatic adenocarcinoma; however, only 20% of patients with pancreatic adenocarcinoma are found to have localized, operable disease at time of diagnosis. Unresectable pancreatic adenocarcinoma, primarily within the head of the pancreas, commonly presents as obstruction of the common bile duct as well as the duodenum, and therefore the goal of treatment is that of palliation. Advanced endoscopic procedures, such as ERCP, have now moved to the forefront in the management of stabilizing localized disease or palliation of advanced disease, given the higher morbidity and mortality with surgical approaches to palliation (i.e., biliary bypass, gastrojejunostomy) [21].

Palliation of Biliary Obstruction

Endoscopic biliary stenting was first introduced in the early 1980s with the use of plastic stents for decompression of biliary strictures. Until then, surgery was the mainstay of therapy. Placement of biliary stents is performed under fluoroscopic guidance once biliary cannulation is successful. Biliary sphincterotomy is typically performed prior to stent placement. The choice of stent will be determined by the length of the biliary stricture, relationship of cystic duct to the common bile duct, and the tumor characteristics and patient prognosis. The questions about durability and patency of plastic stents later fueled the development of metal stents in the late 1980s.

The goals of palliation using biliary stent placement are for symptomatic relief of obstructive jaundice, prevention of cholangitis, and prolongation of survival. Stenting has also been found to improve quality of life. Hyperbilirubinemia has been associated with poor quality of life in patients with malignant biliary obstruction and contributes to jaundice, pruritus, anorexia, and weight loss. Biliary decompression effectively improves QOL due to its improvement in the symptoms related to hyperbilirubinemia [22, 23].

Plastic stents were first designed for biliary decompression of malignant biliary strictures. Plastic stents are composed of polyethylene, polyurethane, or Teflon. Stent diameter ranges from 5 F to 12 F, though 10 F is the standard size used for bile duct obstruction. All plastic stents are radiopaque. Plastic stents are commonly used because of their efficacy and low cost. The primary indication for self-expandable metal stent (SEMS) placement in unresectable pancreatic cancer is for alleviation of

obstruction and improvement in the quality of life in patients with a survival greater than 4–6 months. SEMS are composed of metal alloys (most frequently nitinol) which allow for adequate expansible radial force without sacrificing flexibility and conformability to the duct. When fully expanded, SEMS diameter ranges from 6 to 10 mm. All self-expandable metal stents are radiopaque. Self-expandable metal stents can be covered, partially covered, or uncovered. They are also much more expensive than plastic stents [24].

Complications of biliary stent placement include the complications related to the ERCP itself along with stent-specific complications, including occlusion and migration. Plastic stents have been observed to have increased rates of occlusion compared to SEMS. Bacterial adhesion to the plastic stent with formation of glycoprotein-rich biofilms has been implicated in stent occlusion as well as the relatively small diameter. Occlusion typically occurs at 3–6 months with plastic stents. Stent occlusion is typically accompanied by cholangitis, and therefore exchange of the plastic stent is necessary [25].

Stent occlusion is also seen in metal stents, though seen at a lower rate. With uncovered metal stents, tumor in growth through the metal lattice has been the primary hypothesis for occlusion. Uncovered metal stents are typically unable to be removed or repositioned due to tumor ingrowth, whereas partially covered or fully covered metal stents can be removed with the use of a snare or repositioned if needed. While plastic stent occlusion is managed by stent removal and exchange, in SEMS typically another stent is placed in a stent-in-stent fashion, as SEMS are unable to be removed. Stent migration also poses concern when determining type of stent. Covered metal stents tend to have increased rates of migration compared to partially covered or uncovered metal stents. In a randomized, multicenter trial of 400 patients with unresectable distal malignant strictures, there was no statistical difference in survival, stent patency, or complication rates in covered versus uncovered metal stents; however, stent migration occurred in 3% of patients in the covered metal group compared to no patients in the uncovered metal group ($p = 0.03$) [26]. Lastly, another concern with the placement of plastic stents and fully covered SEMS is the risk of cholecystitis due to stent position in relation to the cystic duct take-off. The rate of cholecystitis after SEMS has ranged from 5% to 11% in many reports. Several risk factors for cholecystitis after SEMS include stent position as well as tumor involvement to the level of the orifice of the cystic duct [27]. Hence, care must be taken to identify the cystic duct take-off prior to stent deployment.

With the background knowledge of the types of stents available and complications related to each type of stent, multiple studies have been done in order to compare stent types to assist the providers in determining the appropriate stent for the right patient. Long-term outcomes of endoscopic palliative stenting have been detailed in the literature. In a retrospective study of 100 patients with unresectable pancreatic adenocarcinoma, common bile duct obstruction occurred in 81 patients. Of those patients, 74 (88%) had successful endoscopic placement of biliary stents. Of the patients who underwent endoscopic stent placement, 59 patients had SEMS placed at first intention with stent occlusion occurring in 31%. The median duration of metallic stent patency was 7 months. In the 15 patients with plastic stents placed,

13 (87%) developed occlusion with a median stent patency of 2.5 months [28]. One of the first major randomized studies done in Sweden was a single-center randomized prospective trial which compared plastic stents to covered SEMS in patients with malignant biliary strictures. One hundred patients were randomized to the plastic stent group or the covered SEMS group with the primary outcome being time to stent failure, as defined by signs and symptoms of cholangitis and rising bilirubin then confirmed by ERCP. The covered self-expandable metal stents were superior to plastic stents in patency times with a median patency of 3.6 months and 1.8 months, respectively. The investigators noted an overall shorter duration of stent patency compared to previous studies and attributed this to the low overall median survival in this study of 4.5 months [29].

When determining which stent is appropriate for which patient, the most suggested approach is to determine the life expectancy of the patient with unresectable pancreatic adenocarcinoma. In patients with shorter life expectancy, about 4 months or less, the mainstay for decompression is with the use of plastic stents. This is the most cost-effective method for quality of life improvement. With a life expectancy of greater than 4 months, no distant metastasis, or even as a bridge to more definitive therapy, SEMS placement is the most effective choice for biliary decompression. Successful placement of biliary stents to relieve malignant biliary obstruction occurs in greater than 90%. In general, patients with a life expectancy of greater than 4 months, SEMS are preferable to plastic stents due to lower stent failure, lower risk of cholangitis, decreased total number of hospitalizations secondary to stent-related complications, and therefore decreased overall cost.

Stenting in Resectable Disease

For localized pancreatic cancer, the goal is for curative therapy with surgery. However, there has been conflicting evidence about what to do with patients with resectable disease who develop symptoms of biliary obstruction. Stenting can relieve symptoms of biliary obstruction (pruritus, cholangitis), but controversy still remains about whether decompression can decrease the morbidity and mortality related to a Whipple procedure. Concerns of preoperative biliary stenting arise impart from the idea that inflammation from SEMS may lead to complications associated with surgical resection [30, 31]. Several studies have demonstrated that preoperative biliary decompression leads to increased complications, particularly infectious complications. In a Cochrane review which included 6 trials of 520 patients that compared preoperative biliary drainage versus no drainage, there was no significant difference in mortality; however, the overall serious morbidity was significantly higher in the preoperative biliary drainage group compared to the no-drainage group (RR 1.66; 95% CI 1.28–2.16; $P = 0.0002$) [32]. This review included all patients with biliary obstruction whether benign or malignant and noted that the results were at high risk of bias. The authors concluded that there was not enough evidence to support or refute the routine practice of preoperative biliary drainage for obstructive jaundice [25]. A retrospective analysis of 593

patients treated with pancreaticoduodenectomy (PD) at Memorial Sloan Kettering analyzed patients who did receive preoperative biliary drainage for pancreatic cancer to determine whether stent type (SEMS versus plastic) made a difference in surgical outcomes. The study revealed that self-expandable metal stents did not affect postoperative complications, 30-day mortality, length of stay, anastomotic leak, margin status, or determination of unresectability at time of resection; however, there were more wound infections and longer operative times observed compared to plastic stents and those who were not stented (wound infection rates, 31% SEMS vs. 12.8% plastic stent vs. 6.2% no stent groups, $p < 0.001$) [30]. In conclusions drawn from a surgical group in Italy studying post pancreaticoduodenectomy wound infections after preoperative biliary stenting, they note the need to reduce the wait time for PD as well as provide antibiotic prophylaxis to prevent incisional and abdominal wound infections [33].

In patients with locally advanced disease who are candidates for neoadjuvant chemotherapy and with symptoms of biliary obstruction, biliary decompression is necessary in order to proceed with chemotherapeutic agents, usually gemcitabine, while awaiting surgical resection. Placement of self-expandable biliary stents during the neoadjuvant period has been shown to be efficacious with lower complication rates compared to plastic stent placement [33–35]. In practice, routine preoperative biliary stenting is not indicated, except in patients who have a delay in pancreaticoduodenectomy for neoadjuvant chemotherapy or with symptomatic hyperbilirubinemia (i.e., cholangitis). Once the decision is made for biliary drainage, SEMS are superior to plastic stents in patency and appear to have no significant impact on resectability or overall serious impact on surgical complications.

Fiducial Placement

Radiation therapy has a role in the management of pancreatic adenocarcinoma, particularly for locally advanced disease and palliation of pain. Stereotactic radiation therapy allows delivery of high-dose beam radiation with pinpoint accuracy to a localized target. The difficulty with radiation therapy in pancreatic adenocarcinoma is the variation with respiratory motion. Fiducial markers are used for localization of the radiation site and to track respiratory motion. Fiducial markers are radiopaque coils or spheres that are implanted into the target lesion and serve as reference for real-time tumor tracking during radiation therapy. Given the excellent visualization of pancreatic adenocarcinoma within proximity to the luminal gastrointestinal tract, endoscopic ultrasound has become a growing method for the placement of fiducial markers. The technique for injection of fiducials is similar to fine needle aspiration and can be delivered with 19- or 22-gauge needles. Several studies have reported an 88–90% success rate of EUS-guided fiducial placement. The complication rate is approximately 2% with reported complications including pancreatitis, minor bleeding, abdominal pain, and elevated liver enzymes. The rate of fiducial migration has been reported to be about 7% [36, 37].

Palliation of Duodenal Obstruction

Advanced pancreatic adenocarcinoma of the head of pancreas can cause invasion into the adjacent duodenum thereby leading to gastric and duodenal obstruction. Symptoms are characterized by intractable nausea, vomiting, abdominal fullness, and early satiety. Duodenal stenosis can occur in 10–25% of unresectable head of pancreas adenocarcinoma and unfortunately is the presenting symptom in 6% of cases [38]. Historically, management of malignant gastric and duodenal obstruction secondary to pancreatic cancer was open surgical bypass which was a procedure with relatively high morbidity and mortality. The advances in endoscopic therapy have provided effective and less morbid means for palliation of gastric outlet obstruction.

Palliation of gastric or duodenal outlet obstruction is done with an enteral self-expandable metal stent (SEMS) with a large diameter of up to 22 mm and 60–90 mm in length. The stent can be flared at the proximal end or at both ends to help reduce the risk of migration. Due to the size of the delivery catheter, a therapeutic endoscope, which has a large working channel, is required. The stent is positioned across the stricture typically with the use of fluoroscopic guidance. Contraindications to enteral stenting include perforation and multiple discrete areas of distal obstruction, which can be due to peritoneal carcinomatosis. Importantly, peritoneal carcinomatosis alone, without obstruction, is not a contraindication to enteral stenting. Most common complications include stent obstruction, migration, and more rarely perforation. Duodenal stenting has been shown to be technically feasible with a technical success rate of 96% and provides clinical relief of symptoms with an 88% efficacy rate [28, 39]. The difference between technical success and clinical success may be, in part, due to alterations in gastrointestinal motility in patients with pancreatic cancer. In a recent prospective, multicenter observational study out of Japan, 39 patients (41% with pancreatic adenocarcinoma) underwent uncovered self-expandable metal duodenal stent placement for gastric outlet obstruction. The clinical success rate was found to be 92% [40]. Researchers in the UK also report a positive experience with SEMS in relieving gastric outlet obstruction in the setting of advanced pancreatic adenocarcinoma. In a small case series of eight patients, stenting was successful in seven patients with a success rate of 88%. All patients were able to tolerate a solid diet upon hospital discharge. There were no complications in this cohort; however, the median survival after stent placement was 10 weeks. This low median survival unfortunately reflects the advanced and aggressive nature of pancreatic cancer once duodenal invasion occurs. Several investigators have also assessed quality of life scores after palliative enteral stenting for gastric outlet obstruction. In a randomized control trial from Mehta et al. 27 patients were randomized to laparoscopic gastrojejunostomy versus duodenal stenting. Length of hospitalization was longer ($p = 0.02$) and postprocedure pain scores were worse ($p = 0.05$) after laparoscopic gastrojejunostomy. After 1 month, patients who received duodenal stents reported significant improvement in quality of life based on physical health questionnaire assessments [41].

Duodenal obstruction can coincide with biliary obstruction in patients with advanced disease. In this case, biliary stent placement should be done during the same procedure as enteric stent placement. Self-expandable biliary stents should be placed prior to duodenal stent because biliary access becomes significantly difficult as the duodenal stent crosses the papilla [39]. Palliation of duodenal obstruction with enteral stenting improves quality of life with less associated morbidity and mortality and has become the method of choice.

Palliation of Pain

Medical management of pain secondary to pancreatic cancer relies on nonsteroidal anti-inflammatory medications, with opioids as the next line. Abdominal pain is a common symptom for pancreatic adenocarcinoma. It is usually chronic, continuous, and dull, often requiring opioids for relief. Opioids can often provide adequate relief but are associated with constipation, sedation, drowsiness, nausea, and vomiting. There are many cases in which pain symptoms become severe and resistant to opioids. It is postulated that refractory abdominal pain is due to tumor invasion into the celiac plexus or neural alterations within the pancreas itself. With EUS guidance, advanced endoscopists have begun to relieve abdominal pain symptoms with celiac plexus neurolysis and blocks. The procedure is technically straightforward since the celiac axis is typically located within a few centimeters of the gastric wall. After identification of the celiac artery take-off from the aorta by endoultrasonography, a solution of absolute alcohol is injected adjacent to the ganglion and is used to permanently ablate neural tissue of the celiac ganglion (neurolysis). Alternatively, a solution of triamcinolone can be used for more temporary analgesia (celiac plexus block) though this is a less suitable option for patients with refractory abdominal pain from pancreatic cancer. In a meta-analysis by Puli et al., the pooled proportion of patients with relief of pancreatic cancer pain after EUS-guided celiac plexus neurolysis was 80.2% [42]. The treatment effect is approximately 4–5 weeks, but reports indicate effects can last up to 3 months. Side effects of celiac plexus neurolysis include bleeding, infection, diarrhea, and hypotension. These complications are commonly associated with direct blockade of the sympathetic efferent activity and are typically minor and self-limited [43]. Rare are reports of paresis or paresthesias. Given its reasonable efficacy and favorable safety profile, early consideration of EUS-guided neurolysis is recommended for patients with unresectable pancreatic adenocarcinoma who have abdominal pain requiring regular use of opiates.

Another component of pancreatic cancer pain is “obstructive pain” which is secondary to upstream dilation of the pancreatic duct (PD) due to a distal pancreatic stricture. This pain typically worsens after meals, similar to chronic pancreatitis. One of the initial case series by Costamagna of 12 patients with obstructive pain who received pancreatic duct stents revealed a technical success rate of 66% and pain resolution occurring in 87% [44]. A subsequent small prospective study enrolled 20 patients with unresectable pancreatic cancer with PD obstruction and postprandial

abdominal pain. Plastic pancreatic duct stents were placed. Pain scores decreased by three points at 4 weeks ($p < 0.001$) and quality of life scores also improved at 4 weeks ($p < 0.01$) [45, 46]. Thus, in a selected group of patients with obstructive pain symptoms, pancreatic duct stenting may be an alternative and safe measure for palliation.

Conclusion

Advanced endoscopy, including the use of ERCP and EUS, has become a key tool in the diagnosis, treatment, and palliation of pancreatic cancer. Diagnosis and management of pancreatic cancer require a multidisciplinary approach. The difficulty with diagnosis has driven the field of therapeutic endoscopy to find alternative methods from routine brush cytology, to the use of cytogenetics and molecular analysis to direct visualization imaging modalities and biopsy. With advances in endoscopy and ERCP in particular, there has been an evolution from surgical or percutaneous biliary decompression to endobiliary stent placement, leading to relief of obstructive jaundice, reduction of pain, and improved quality of life. Advanced endoscopists should be an active member in the care of patients with pancreaticobiliary malignancy in all stages of disease.

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Interventional Radiology for Pancreatic Cancer

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Abstract

Pancreatic adenocarcinoma is the 12th most common malignancy globally, holding joint position with renal cancer, and is the seventh leading cause of cancer-related mortality and second most common cause of cancer deaths for all gastrointestinal-related carcinomas. Most patients present late in their course and have either locally extensive or metastatic disease with a median survival of only 4–6 months. At the time of diagnosis, unfortunately only 10–20% of patients are candidates for curative resection. The late presentation, aggressive nature, and lack of effective therapies all contribute to the poor prognosis. It is typical that these patients with more advanced disease will undergo either interventional radiology (IR)- or endoscopic ultrasound (EUS)-guided interventions to deliver either preoperative or palliative care. The objective of this chapter is to highlight currently available and emerging IR- and EUS-guided interventions as they apply to the care of patients with pancreatic carcinoma.

Keywords

Endoscopic ultrasound · Endoscopic retrograde cholangiography-guided drainage · Endoscopic ultrasound-guided bile duct drainage · Duodenal stenting · Celiac plexus and ganglia neurolysis · Ablative antitumor therapies

Introduction

During the first 75 years of the American Roentgen Ray Society (ARRS), which was the first established radiology society in the United States, the field of interventional radiology (IR) was viewed largely as a rogue practice that dealt with theoretical concepts and practices. Angiographers were the pioneers of the field, but were often viewed as heretics by surgeons, and their practice was seen as time-consuming and of minimal utility and productivity by radiology colleagues. Early interventions were limited and restricted to the care of bleeding lesions and hypervascular tumors [1]. The advent of modern IR suites containing mobile multi-angle fluoroscopy C-arms, the capability of digital image acquisition, and the development of an array of dedicated accessories fostered the development of new techniques that are applied to a growing number and broader spectrum of diseases.

Similarly, endoscopic ultrasound (EUS) has experienced an evolution in its role since its introduction in 1980. From that time until the mid-1990s, EUS was utilized solely as a diagnostic imaging modality, providing greater diagnostic sensitivity than transabdominal ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), or positron emission tomography for most benign and malignant pancreatic disorders [2–6]. With the development of linear array imaging, it became possible to obtain tissue samples under real-time ultrasound guidance permitting fine needle aspiration (FNA) with cytological evaluation and core biopsy histological architecture assessment, which further enhanced diagnostic accuracy [7, 8]. More recently, EUS has been utilized to guide therapeutic interventions for an array of pancreatic disorders.

Interventional radiology- and EUS-guided interventions may be applied with diagnostic and/or therapeutic intent for a spectrum of benign and malignant pancreatic diseases. The objective of this chapter is to review these interventions and to focus more fully on EUS-guided therapies and to discuss the various techniques, their role, and available data as applied specifically to the management of patients with pancreatic carcinoma.

Bile Duct Drainage

Malignant biliary obstruction is most commonly associated with pancreatic carcinoma and develops in 70–90% of patients often resulting in jaundice, pruritus, hepatocellular dysfunction, cholangitis, malabsorption, and coagulopathy [9–11]. Biliary decompression may be achieved by endoscopic retrograde cholangiography (ERC), interventional radiologic or surgical means. These techniques are equally effective at relieving jaundice with no difference in overall survival [10–19]. Endoscopic stent insertion safely and effectively reestablishes bile flow, alleviates jaundice and pruritus, and may improve quality of life (QOL) [10–22]. In addition, ERC and stent placement may offer lower morbidity and mortality, shorter hospitalization, and diminished overall cost compared to radiologic or surgical approaches [10, 13–15, 19]. Therefore, in most centers, ERC is favored for palliation of malignant biliary obstruction in patients who require neoadjuvant therapy as a bridge to surgery or for patients with unresectable disease resulting from extensive locoregional spread or distant metastases as a palliative intervention.

Endoscopic Retrograde Cholangiography (ERC)-Guided Drainage

Endoscopic insertion of plastic or metal stents is technically successful in about 90–95% of patients with malignant biliary obstruction [23–27]. Plastic stents are commonly used due to their efficacy and low cost (Fig. 1). These stents are easily exchanged as long as duodenal narrowing does not prohibit passage of the endoscope. The major drawback of plastic stents is the formation of a bacterial biofilm leading to stent obstruction, recurrent jaundice, and occasional cholangitis. As a result, repeat ERC and stent exchange are necessary in about 30–60% of patients [10, 18, 23, 25, 27, 28]. Efforts to prolong plastic stent patency have included alterations in stent design and administration of ursodeoxycholic acid, antibiotics, aspirin, or other agents [29–32]. Unfortunately, these therapies have had minimal impact on stent patency and clinical outcomes. More recently, self-expanding metal stents (SEMS), which achieve a larger luminal diameter, have been used with the goal of prolonging stent patency (Fig. 2). Comparative trials demonstrate greater patency and overall cost-effectiveness for SEMS relative to plastic stents, due to the need for fewer repeat interventions [23–27]. However, they offer no survival advantage compared to plastic stents and have an uncertain influence on quality of life [23–27]. Therefore, the selection of plastic versus SEMS for the relief of malignant

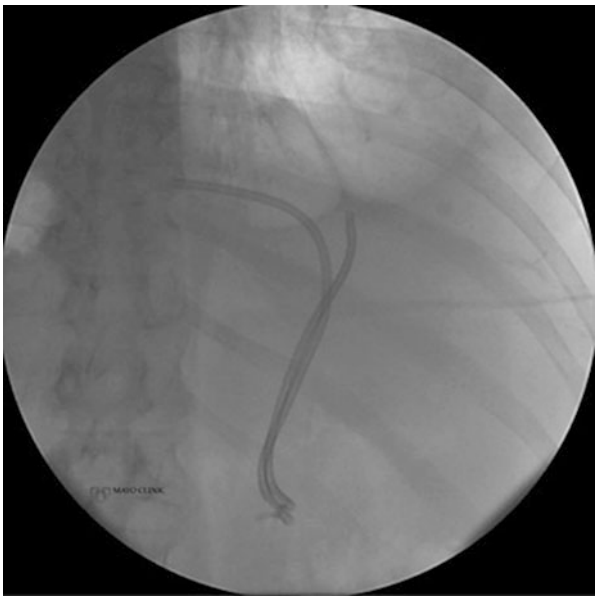

Fig. 1 A fluoroscopic image demonstrates the deployment of two plastic stents that provide drainage for malignant biliary obstruction

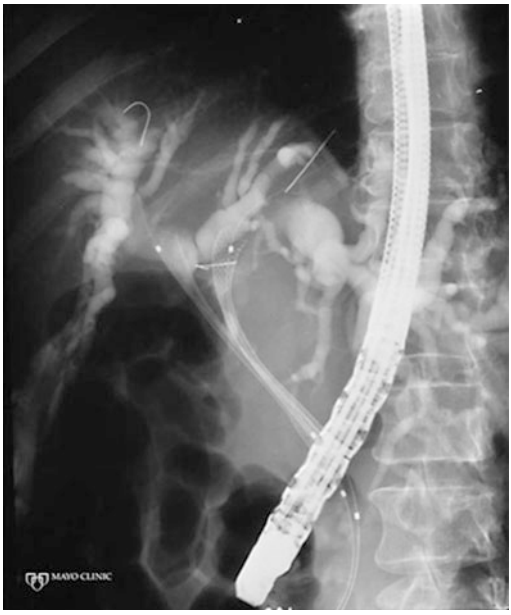

Fig. 2 A fluoroscopic image demonstrates the deployment of two metal stents that provide drainage for malignant biliary obstruction

extrahepatic biliary obstruction is currently debated. However, most agree that for patients with biopsy-proven unresectable malignant obstruction as a bridge to surgery while receiving neoadjuvant therapy or poor operative candidates, with >6 months of expected survival, the weight of evidence favors initial insertion of a SEMS (Fig. 3). In this setting, the prolonged patency provided by SEMS compared

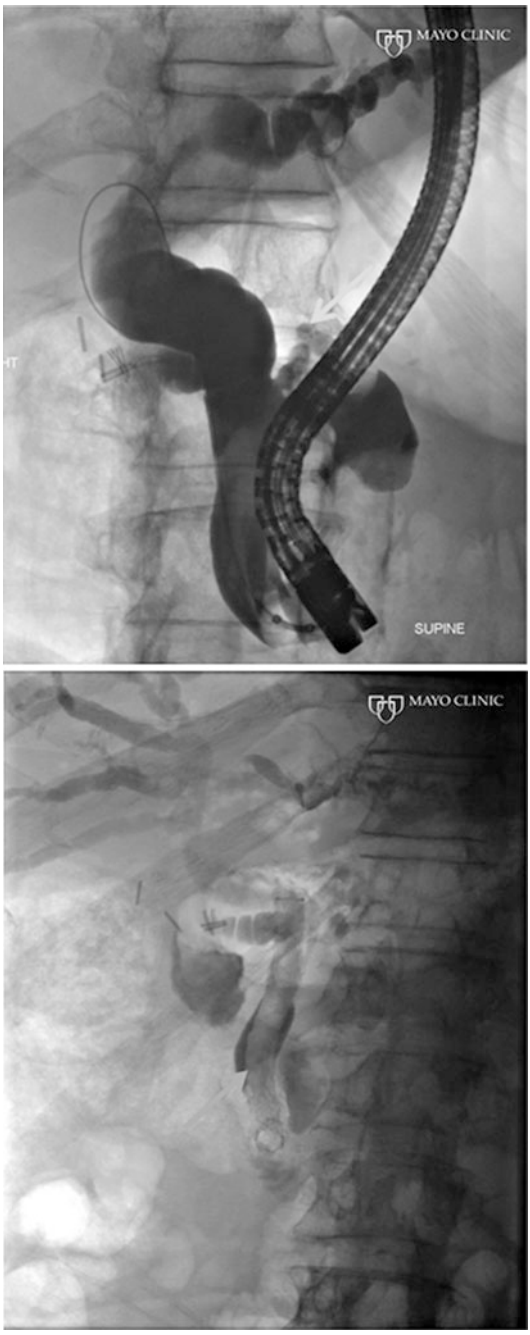

Fig. 3 A patient with malignant transformation of IPMN presented with jaundice secondary to passage of mucous via a fistula tract that developed from the pancreatic duct and tumor into the biliary tree (*top image*). A covered metal stent was placed to inhibit the flow of mucous resulting in marked improvement in the biliary obstruction (*bottom* image)

to plastic stents favors their use for patients with relatively prolonged expected survival because of the potential to obviate the need for repeat ERC and stent exchange. For patients with expected survival of 6 months or less, the literature supports placement of a plastic stent [33]. The principles of stent patency and selection appear to apply regardless of the means of access or deployment.

Interventional Radiology (IR)-Guided Drainage

ERC may not provide drainage secondary to failed cannulation, presence of severe tumor-induced stricturing of the bile duct and/or duodenum, or an inaccessible papilla secondary to congenital or surgically altered anatomy. In the case of failed ERC, depending on the center, interventional radiology may be employed to perform a percutaneous transhepatic biliary drainage (PTBD). PTBD was initially described more than three decades ago and is usually performed using transabdominal ultrasound guidance [34]. An intrahepatic bile duct branch is accessed usually with an 18–22-gauge needle and a guidewire is inserted over which a 7–10-Fr catheter is placed under fluoroscopic guidance. Puncture with a smaller caliber (22-gauge) needle has been shown to be safer in patients without intrahepatic bile duct dilatation. Drainage is successful in approximately 95% of patients with dilated intrahepatic bile ducts, but only 70% of patients with non-dilated ducts [35]. Complications develop in as many as 32% of patients undergoing PTBD including cholangitis, fistula formation, peritonitis, empyema, and liver abscess [36, 37]. Percutaneous metal stent placement provides comparable palliation regardless of tumor site (proximal vs. distal) and irrespective of tumor type. Studies demonstrate that for extrahepatic malignant obstruction, the duration of stent patency is comparable whether the stent traverses the malignant stricture and papilla as compared to a position proximal to, and upstream from, the site of obstruction [38].

Endoscopic Ultrasound (EUS)-Guided Drainage

EUS is a more recently introduced method for providing bile duct drainage following failed ERC and provides an alternative to IR and surgical approaches. Depending on the procedure indication, patient anatomy, and scope access, one may use EUS to perform either transpapillary or transanastomotic drainage versus transluminal drainage in the form of either a hepaticogastrostomy or choledochoduodenostomy. EUS is ideally performed with a “therapeutic” linear array echoendoscope that contains a larger caliber channel thereby allowing the use of a greater array of accessories and insertion of larger (up to 10-Fr) diameter stents. Smaller caliber “diagnostic” echoendoscopes may be used to perform a rendezvous wire passage or for placement of 7-Fr or smaller stents.

The most common indication for EUS-guided biliary tree access and therapy is following failed efforts at standard endoscopic (ERC) techniques, for access and drainage may occur as a result of:

1. Underlying pathology, including inflammation, tumor, stricture, stone, etc., that prohibits biliary and/or gastrointestinal luminal access or traversal
2. Anatomical variants such as a duodenal diverticulum, pancreas divisum, or a disrupted duct
3. Surgically altered anatomy resulting from pancreaticoduodenectomy or anastomotic stricture

These techniques are also performed following failed, or instead of, percutaneous efforts for biliary and standard endoscopic (ERC) techniques, for access and drainage may occur as a result of caliber channel thereby allowing ultrasound for poor operative candidates.

Transpapillary/Transanastomotic Drainage

Transpapillary drainage is possible only when a guidewire can be advanced through the site for biliary and standard endoscopic (ERC) techniques, for access and drainage may occur as a result of completion of the rendezvous portion of the procedure. Similar techniques and principles apply to transanastomotic drainage, which may be necessary to evaluate for tumor recurrence or complications following pancreaticoduodenectomy.

The procedure involves positioning the echoendoscope within the stomach or duodenum in a manner that allows traversal of the least amount of tissue to access the desired duct. The echoendoscope is typically placed within the duodenal bulb when accessing the extrahepatic bile duct or within the stomach (cardia, fundus, or proximal body) when accessing an intrahepatic bile duct. Concurrent EUS imaging allows one to exclude the presence of intervening structures such as blood vessels and any undesired ducts. A needle is advanced under EUS guidance preferentially into a dilated duct and one that lies in a longitudinal (or parallel) orientation in order to facilitate access, passage of accessories, and device deployment. Typically either a 19- or 22-gauge needle is employed. Larger caliber needles allow the use of a larger gauge and stiffer wire that facilitates traversal of stenotic strictures and passage of accessories. However, initial duct access can be more difficult when using a larger gauge and stiffer needle. A clear understanding of the procedure goals can help guide equipment selection. For example, it may be reasonable to use a 25-gauge needle if the intended goal is to only obtain a cholangiogram. Some also prefer the smaller gauge needle to determine if contrast freely flows into the anastomosed bowel lumen suggesting the absence of critical stenosis, thereby potentially obviating the need for therapeutic intervention (e.g., anastomotic dilation and stenting). However, this practice is controversial and some instead advocate noninvasive imaging modalities such as magnetic resonance imaging (MRI) and cholangiopathy (MRCP).

Duct access is confirmed by aspirating bile and performing cholangiography, which also allows delineation of the anatomy. Next a guidewire is advanced in an antegrade fashion across the site of obstruction and papilla under fluoroscopic guidance and then coiled within the small bowel to reduce the risk of dislodgement that may occur with removal of the echoendoscope or during insertion of the duodenoscope. Care must be taken to minimize the risk of wire shearing that may

result from manipulation of the wire and abrasion against the sharp needle tip. The selection of guidewire caliber is based on the needle caliber. The use of a 19-gauge needle allows the use of 0.035 in guidewires or smaller, whereas 22-gauge needles can only accommodate 0.018 in guidewires. One cannot automatically assume that a needle of a particular gauge, or wire of a particular caliber, can replace a similarly sized needle or wire, because of the minor variation that exists in equipment among companies [39].

Depending on the echoendoscope orientation, access from the duodenal bulb often results in passage of the guidewire into the proximal (intrahepatic) ducts rather than distally through the papilla. This problem can usually be overcome by altering the scope position and/or by elevator deflection. Alternatively, the guidewire may be intentionally advanced into the intrahepatic biliary tree to induce looping and eventual passage in the alternate direction toward the papilla. Likewise, access to the left intrahepatic bile duct often leads to inadvertent passage to the right intrahepatic ductal system instead of the intended extrahepatic bile duct, sometimes overcome by altering the angle of entry or by intentionally looping within the right system and eventually distal migration.

Once the guidewire is adequately positioned, the echoendoscope is back-loaded leaving the guidewire in place and the rendezvous portion of the procedure is performed. To do so, a standard forward-viewing or side-viewing duodenoscope is advanced alongside to the guidewire and down to the papilla or site of anastomosis. A snare or biopsy forceps is used to grasp the guidewire, which is then withdrawn through the accessory channel. Care must be taken when grasping and retracting the guidewire due to the resulting tension that may be placed on the wire and resulting risk of severing tissues traversed by the wire. Thereafter, the ERC (retrograde) portion of the procedure, including dilation (catheter or balloon) and stent placement, is performed in standard fashion. In patients requiring transanastomotic drainage, for instance, within an afferent jejunal limb or Roux-en-Y reconstruction after pancreaticoduodenectomy, the ERC is typically performed with either a pediatric colonoscope or a prototype long, oblique-viewing enteroscope [39].

Transluminal Drainage

Transluminal stenting indicates the creation of a trans-enteric fistula with placement of a stent across the gastric wall into an intrahepatic bile duct (hepaticogastrostomy) or across the duodenal bulb wall and into the extrahepatic bile duct (choledochoduodenostomy) via a suprapancreatic or intrapancreatic route. These techniques are required when the guidewire cannot be advanced through the site of obstruction or papilla or when an endoscope cannot be advanced into the small bowel to allow guidewire retrieval.

While many of the aforementioned techniques and principles for EUS-guided transpapillary drainage also apply to transluminal drainage, there are some notable differences. The goal of EUS-assisted portion of transpapillary drainage was simply to provide guidewire insertion to allow the subsequent rendezvous portion of the exam (Fig. 4). Therefore, transpapillary drainage does not require dilation of the tract from the gut wall to the bile duct and intervening tissues. In this circumstance, a

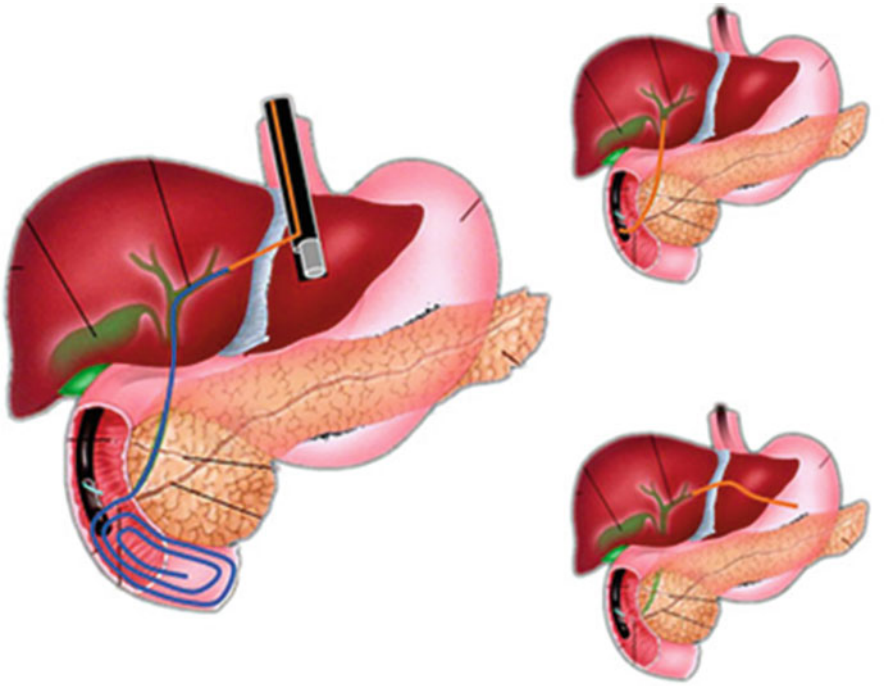

Fig. 4 Aspects of EUS-guided rendezvous procedures. The image to the *left* demonstrates EUS-guided access to a left intrahepatic bile duct with guidewire passage through the papilla and coiled within the duodenum as may be necessary following failed endoscopic retrograde cholangiography. Doing so allows subsequent transpapillary drainage (*right*, *top*). When transpapillary drainage cannot be achieved, then hepaticogastrostomy with stent placement through the stomach to an intrahepatic bile duct provides an alternative means of drainage (*right*, *bottom*)

smaller channel diagnostic echoendoscope is sufficient. Transluminal drainage, on the other hand, does necessitate tract dilation and benefits from the use of a larger caliber echoendoscope thereby allowing the use of a broader range of accessories and deployment of larger caliber stents. A variety of standard biliary and pancreatic catheter dilators and balloon dilators may be used with selection based on the patients' anatomy. Equipment use varies among endoscopist and may require trial and error and often necessitates use of multiple devices. Following tract dilation, a stent is advanced through the gut lumen and into the biliary tree. There are also reports of EUS-guided antegrade stent insertion directly into a dilated bile duct via the gastric wall [40].

Technical Success, Outcomes, and Complications (For EUS-Guided Bile Duct Drainage)

There is a relative paucity of data regarding EUS-guided bile duct drainage, and they arise from studies that employed varying techniques and sometimes limited methodology (Figs. 5 and 6). Therefore, it is not possible to firmly establish the technical success

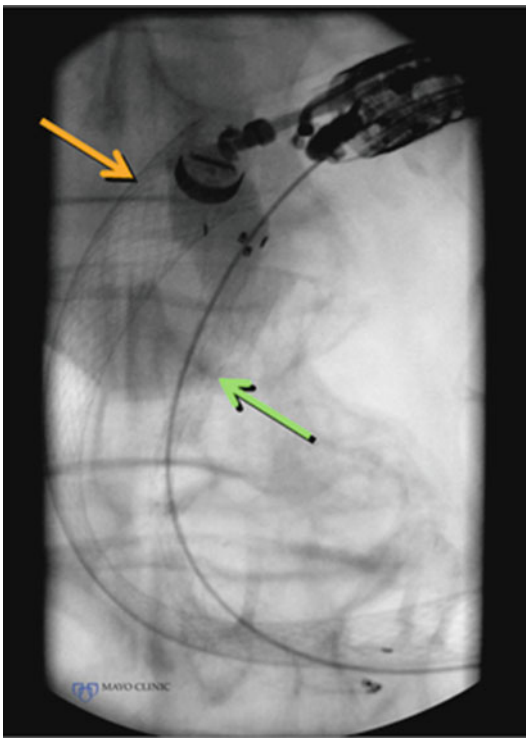

Fig. 5 A patient presented with an indwelling duodenal stent (*orange arrow*) to help alleviate duodenal obstruction secondary to pancreas cancer. Given that the papilla was no longer accessible and following two failed efforts at ERC, the patient was referred for EUS-guided bile duct access with a stent placed (*green arrow*) via an EUS scope in an antegrade fashion

and complication rates. It is even more difficult to verify the clinical success and role of these techniques based on current data when evaluating the collective literature [1996–2008, $n = 92$ patients]; it appeared that EUS-guided biliary access, either transhepatic or extrahepatic, had a 79% technical success rate (Table 1) [39–52]. Available data suggest that pain relief was experienced in approximately 65% of patients in whom this served as the primary indication for the procedure. The impact on other clinical features such as recurrent pancreatitis or steatorrhea cannot be discerned.

However, with the advent of the development of a lumen-apposing metal stent, there are now some preliminary case reports and series highlighting the possible role in gallbladder drainage for patients ineligible for operative intervention [53–55].

These reports also indicate a complication rate of 14% for patients undergoing EUS-guided biliary intervention. Complications included a bile leak ($n = 3$, one patient with a biloma), cholangitis ($n = 2$), pneumoperitoneum ($n = 3$), and one patient each developing pancreatitis, peritonitis, hemorrhage, ileus, and phlegmonous cholecystitis. The duration of follow-up among these studies is too brief to clearly establish the need and timing of re-intervention and long-term outcomes, and many of the studies did not address this issue. The data, however, suggest that stent migration and/or occlusion developed in approximately 20–55% of patients during an often short duration of follow-up. Yamao noted that stents occluded at a time between 4 weeks and 4 months post-procedure [48].

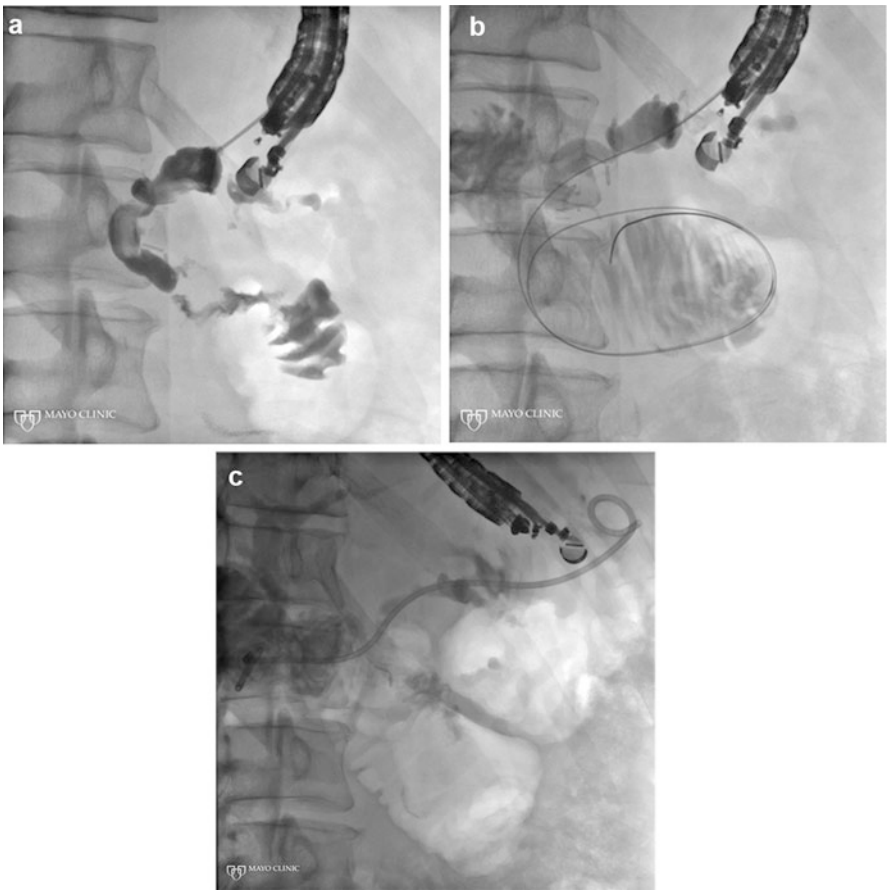

Fig. 6 (**a**–**c**) EUS pancreatitis rendezvous procedure: a patient who had undergone prior pancreaticoduodenectomy for pancreatic cancer presented with recurrent acute pancreatitis felt secondary to a pancreaticojejunal anastomotic stricture. After two failed efforts to identify the anastomosis with a forward-viewing instrument, the patient was referred for EUS-guided therapy. EUS-guided injection revealed a dilated pancreatic duct and anastomotic stricture (**a**). A guidewire was advanced from the stomach, into the pancreatic duct, and coiled within the small bowel (**b**). After balloon dilation, a stent was inserted along the same path (**c**)

Duodenal Lumen Stenting

Patients with pancreatic carcinoma often suffer from gastric retention manifested by bloating, early satiety, and weight loss. This may result from tumor-induced luminal obstruction and/or dysmotility. Palliative surgical intervention for malignant duodenal obstruction is often associated with a significant morbidity. Duodenal stenting has become a popular treatment in cases of malignant stenosis and may be performed by interventional radiologists or endoscopists. These techniques are typically

Table 1 EUS-guided bile duct drainage

Author, year	No. of patients/ interventions	Ductal route	Technical success/ procedure	Complications	Specific adverse events[a]
Wiersema et al. [41, 79]	10	Transhepatic/ extrahepatic bile duct	7/10	1	Pancreatitis
Giovannini et al. [42]	1	Extrahepatic bile duct	1/1	0	N/A
Burmester et al. [43]	4	Transhepatic/ extrahepatic bile duct	3/4	1	Bile leak
Mallery et al. [39]	2	Transhepatic/ extrahepatic bile duct	1/1	0	N/A
Kahaleh et al. [44]	5	Extrahepatic bile duct	4/5	1	Peritonitis
Lai et al. [45]	1	Extrahepatic bile duct	1/1	0	N/A
Puspok et al. [46]	6	Transhepatic/ extrahepatic bile duct	5/6	1	Subacute phlegmonous cholecystitis
Kahaleh et al. [47]	23	Transhepatic/ extrahepatic bile duct	18/23	4	Pneumoperitoneum ($n = 2$), bile leak, minor bleeding
Yamao et al. [48]	2	Extrahepatic bile duct	2/2	0	N/A
Bories et al. [40]	11	Transhepatic	10/11	3	Ileus, biloma, cholangitis
Will et al. [49]	8/10	Extrahepatic bile duct	9/10	1	Cholangitis
Ang et al. [50]	2	Extrahepatic bile duct	2/2	0	N/A
Fujita et al. [51]	1	Extrahepatic bile duct	1/1	0	N/A
Yamao et al. [52]	5	Extrahepatic bile duct	5/5	1	Pneumoperitoneum ($n = 1$)
Tarantino et al. [56]	9	Extrahepatic bile duct	4/9	0	N/A

[a]Excludes stent occlusion and transient post-procedure pain

performed under fluoroscopic guidance and have proven a safe, effective, and less invasive alternative to surgical bypass for managing patients with malignant gastroduodenal outlet obstruction. However, because of the risk, modest impact on clinical endpoints, and cost, there is some debate as to the role and patient selection for these less invasive techniques relative to surgical bypass.

Interventional Radiology (IR)-Guided Duodenal Stenting

Duodenal stenting when performed via interventional radiology involves fluoroscopically assisted catheter and guidewire placement through the esophagus and stomach and eventually traverses the site of malignant duodenal obstruction. Water-soluble contrast is injected to demonstrate the upper and lower aspects of the stricture followed by insertion of a self-expanding metal stent (SEMS). No standard exists regarding catheter, guidewire, or stent selection in terms of length and caliber. Following deployment, contrast is injected to ensure luminal patency and free flow beyond the stent.

Endoscopic Ultrasound-Guided Duodenal Stenting

Endoscopically guided stenting adopts many of the same techniques except for the delivery of devices via a standard forward-viewing endoscope or side-viewing duodenoscope [57]. Initially an endoscopy is performed to delineate the site, degree, and length of obstruction. The length and number of stents used are based on the length of stricture, with the intent that at least 2 cm of additional stent length is on each side of the stricture (Fig. 7). In some cases, multiple overlapping stents may be deployed in a "stent-within-stent" fashion to achieve complete coverage of the stricture. When the nature of the stricture is difficult to interpret by endoscopic visualization, water-soluble radiographic contrast may be injected under fluoroscopic guidance through a catheter passed through the endoscope to define the stricture characteristics. The majority of patients are able to tolerate some oral intake within 24 h of the procedure, and patients should be able to fully resume eating within 7 days. A systematic review of endoscopic SEMS placement for malignant duodenal obstruction analyzed data from 32 case series that included 606 patients [58]. They found that stent placement and deployment was successful in 97% of patients. Clinical success, although variably defined among studies, was achieved in 87% of patients. Disease-related factors accounted for the majority of clinical failures. Subsequent resumption of oral intake was possible for all patients in whom stent deployment was successful, with 87% of patients tolerating soft solids or a full diet, with final resolution of symptoms occurring at a mean of 4 days. There was no procedure-related mortality and the mean survival was 12 weeks. Severe complications (bleeding and perforation) were observed in 1.2% of patients and stent migration was reported in 5%. Stent obstruction developed in 18%, mainly due to tumor infiltration. A more recent prospective evaluation of 51 patients undergoing duodenal stent placement reached

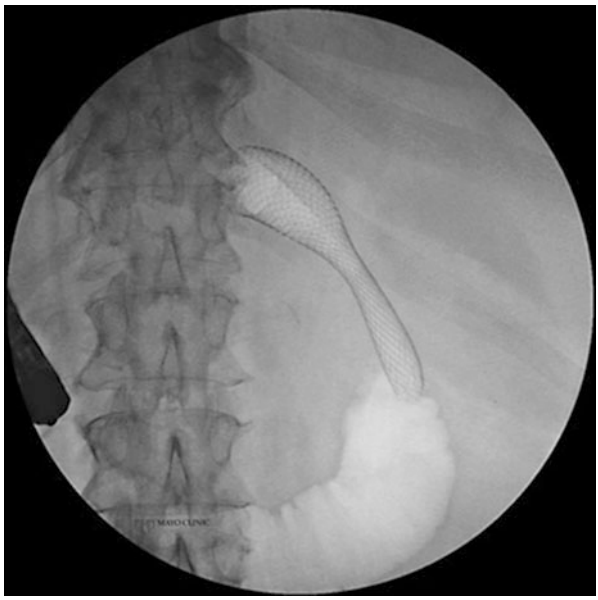

Fig. 7 A fluoroscopic image of a self-expanding metal duodenal stent placed for duodenal obstruction

similar conclusions and demonstrated the feasibility even for those patients requiring concurrent biliary stenting [59]. Priority is often given to first performing biliary stenting prior to duodenal stenting due to the risk and outcome of an uncompressed biliary tree relative to that of gastric retention. However, experience demonstrates that biliary access and drainage may often be achieved either endoscopically or via IR by working through the mesh of a previously placed duodenal SEMS [60]. Similarly, initial placement of a metal duodenal stent proximal to the papilla can allow subsequent biliary access and drainage if necessary (Fig. 8).

Celiac Plexus and Ganglia Neurolysis

Pancreatic cancer commonly produces pain that is difficult to control [61, 62]. Initial therapy with nonsteroidal anti-inflammatory agents is often inadequate and necessitates opioid administration. Although opioids effectively relieve pain, they are associated with a dry mouth, constipation, nausea, vomiting, drowsiness, delirium, and impaired immune function [63, 64]. Therefore, non-pharmacologic therapies, such as celiac plexus neurolysis (CPN), are often given with the goal of improving pain control and quality of life while reducing the risk of drug-related side effects.

Although the terms "celiac plexus" and "splanchnic nerves" are often used interchangeably, they are anatomically distinct structures [65–67]. The splanchnic nerves are located cephalic to the diaphragm (retrocrural), anterior most often to the 12th thoracic vertebra. The celiac plexus is located caudal to the diaphragm (antecrural), surrounds the origin of the celiac trunk, and is comprised of a dense network of ganglia and interconnecting fibers. Ganglia vary in number [1–5], size (diameter

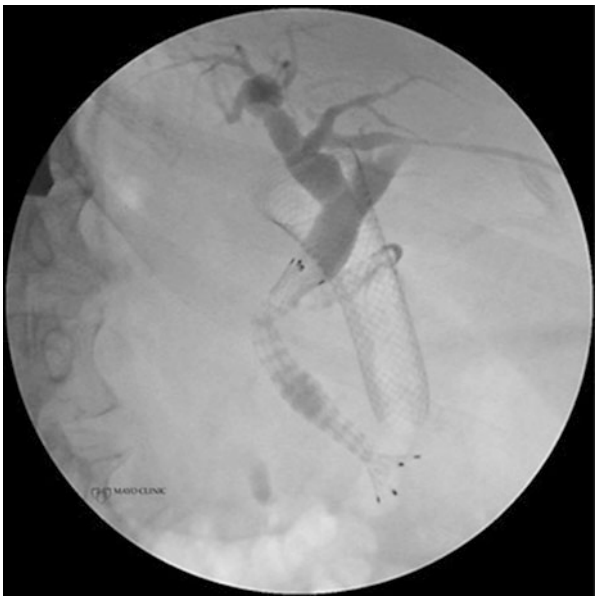

Fig. 8 A fluoroscopic image displaying an example of combined duodenal and biliary metal stent placement in a patient presenting with both duodenal and biliary obstruction

0.5–4.5 cm), and location (T12-L2) [65]. The celiac plexus transmits the sensation of pain for the pancreas and most of the abdominal viscera [68]. The nerves that supply the pancreas can receive nociceptive stimulation and then transmit this pain information to the celiac plexus [69, 70]. Stimuli reach the thalamus and cortex of the brain and this information is perceived as pain. Descending inhibitory mechanisms may also modulate the ascending pain information.

Percutaneous-Guided Celiac Plexus Neurolysis

Kappis described the classic technique in 1914 [71]. Modifications have been created in an attempt to improve the accuracy of needle placement and pain relief, while reducing procedure-related complications. These techniques differ with respect to the route of needle insertion, use of radiologic guidance versus a blind procedure, and chemical composition of the injectate.

For CPN in cancer patients, the injectate usually includes a local anesthetic (bupivacaine or lidocaine) and neurolytic agent (phenol or alcohol). The local anesthetic reduces the discomfort caused by the neurolytic agent. Phenol produces minimal pain because of its local anesthetic effect. Although direct comparisons between alcohol and phenol have not been performed, alcohol is favored because it induces greater neurolysis and presumably greater pain relief [72].

Three meta-analyses have reached conflicting conclusions regarding PQ CPN for intra-abdominal malignancy [73–75]. Lebovits et al. concluded that CPN leads to very successful relief of pancreatic cancer pain [73]. Sharfman et al., on the other hand, found the data insufficient to judge the efficacy, long-term morbidity, or

cost-effectiveness [74]. Most recently, Eisenberg et al. reviewed the literature from 1966 to 1993 including 24 studies, of which 2 were randomized controlled trials, 1 was prospective, and 21 were retrospective uncontrolled trials [75]. The cancer type was specified in 1,117 patients (63% pancreatic, 37% non-pancreatic). Good to excellent pain relief was reported in 89% of patients during the first 2 weeks following CPN. Partial to complete pain relief was reported in about 90% of patients at 3 months and 70–90% at the time of death. Interestingly, pain relief was not influenced by the technical approach or the use of radiologic guidance. The most common side effects, local pain (96%), diarrhea (44%), and hypotension (38%), were generally mild and transient. The authors concluded that (1) CPN has long-lasting benefit for 70–90% of patients with pancreatic and other intra-abdominal cancers, regardless of the technique used, and (2) adverse effects are common but generally transient and mild.

More recently, a prospective, randomized, double-blind study of 24 patients with pancreatic cancer who underwent PQ CPN was reported [76]. The CPN group had a significant reduction in analgesic consumption and drug-induced side effects versus patients treated with drugs alone. Kawamata et al. showed that CPN results in less deterioration in quality of life for pancreatic cancer patients when added to morphine therapy compared to morphine therapy alone or NSAIDs alone, due to the increased duration of the analgesic effect and reduced opioid side effects [77]. A further double-blind, randomized clinical trial assigned 100 patients to receive either CPN or systemic analgesic therapy alone with a sham injection [78]. The CPN and optimized systemic analgesic therapy alone can provide effective analgesia, though CPN can provide significantly better analgesia than optimized systemic analgesic therapy alone. The report also highlighted that CPN had no effect on opioid consumption, quality of life, or survival.

Major complications develop in about 1–2% of patients and include lower extremity weakness and paresthesia, paraplegia, puncture of adjacent organs, and chronic gastroparesis and diarrhea [67, 75, 79]. Neurologic complications result from spinal cord ischemia or direct injury to the spinal cord or somatic nerves. Spinal cord ischemia may result from thrombosis or spasm of the artery of Adamkiewicz located on the left of the spine between T8 and L4, which perfuses the lower two-thirds of the spinal cord [80, 81]. Despite theoretical advantages of given methods, it is believed that the risk of neural dysfunction is not influenced by the technical approach. Paraplegia has been reported with each PQ method regardless of the use of radiologic guidance. There are even several reports of paraplegia following the most direct approach (surgical neurolysis) [82].

Endoscopic Ultrasound-Guided Celiac Plexus Neurolysis

More recently, EUS CPN has been developed for the purpose of enhancing needle localization and spread of the injectate [83]. By doing so, one hopes to minimize complications and improve pain relief. Patients are questioned regarding allergies and the use of anticoagulants. Informed consent is obtained with specific attention to

the unique complications associated with CPN/CPB. Patients are initially hydrated with 500–1,000 ml normal saline to minimize the risk of hypotension. Patients are placed in the left lateral decubitus position and sedated using medications such as midazolam, meperidine, and in some cases droperidol. Throughout the procedure, patients are continuously monitored by an automated noninvasive blood pressure device and pulse oximeter.

Linear array endosonographic imaging from the posterior lesser curve of the gastric fundus allows identification of the aorta, which appears in a longitudinal plane. The aorta is traced distally to the celiac trunk, which is the first major branch below the diaphragm. Color Doppler is used to confirm the vascular nature of the structures. A 22-gauge needle is primed with saline to remove air and then placed through the biopsy channel and affixed to the hub. The needle is inserted under EUS guidance immediately adjacent and anterior to the lateral aspect of the aorta at the level of the celiac trunk, which is the general vicinity of the celiac plexus (Fig. 9). The needle is flushed with 3 ml of normal saline to remove any tissue acquired during insertion. An aspiration test may be performed to rule out vessel penetration prior to injection. Typically, 10 ml (0.25%) of bupivacaine is injected followed by 10 ml (98%) dehydrated alcohol. The alcohol, which produces an echogenic cloud, may lead to discomfort despite sedation. Before withdrawing the needle, it may be flushed with 3 ml normal saline to minimize seeding of the needle track with alcohol, which may produce transient severe post-procedure pain. The entire process is then repeated on the opposite side of the aorta. Occasionally, altered anatomy resulting from significant lymphadenopathy and/or bulky tumors may necessitate injection of the entire solution into one "unilateral" site. The efficacy of "unilateral" versus "bilateral" injection has never been well studied, but data suggest equivalency. After the procedure, which takes about 15 min, the vital signs are monitored for 2 h prior to discharge; patients' blood pressure is checked in both a supine and erect position to assess for orthostasis. CPN is routinely performed as an outpatient procedure, rarely necessitating hospitalization.

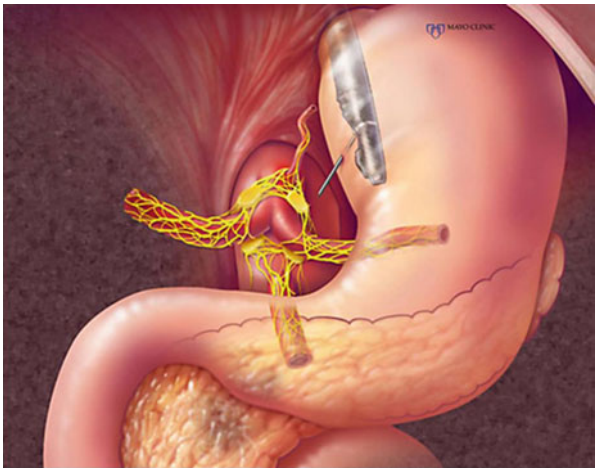

Fig. 9 Illustration demonstrates needle placement adjacent and anterior to the lateral aspect of the aorta at the level of the celiac trunk when performing standard celiac plexus neurolysis

Wiersema et al. published the initial study evaluating EUS CPN [83]. The same group published a follow-up study that included all 25 patients with pancreatic cancer from their initial report [84]. This later prospective study involved 58 patients who underwent EUS CPN for pain secondary to inoperable pancreatic cancer. They injected 3–6 ml (0.25%) bupivacaine and 10 ml (98%) alcohol into both sides of the celiac region. Pain scores were assessed using a standardized 11-point visual analog scale. Forty-five patients (78%) experienced a drop in pain score after EUS CPN. The overall pain scores were significantly lower 2 weeks after the procedure ($p < 0.0001$). Multivariate analysis revealed that sustained pain relief for 24 weeks was independent of morphine use or adjuvant therapy. However, patients who received chemotherapy alone or chemotherapy plus radiation experienced pain relief in addition to that offered by EUS CPN. Pain relief resulting from adjuvant therapy increased over time and at 24 weeks was statistically significant ($p = 0.002$). Although opioid administration increased throughout the study, the increase was not statistically significant. There were no major complications. Minor complications were mild and transient and included hypotension (20%), diarrhea (17%), and pain exacerbation (9%).

Despite 45 patients (78%) experiencing a reduction in pain score, only 31 (54%) experienced a decline of greater than two points, which is a measure of improvement that some consider necessary to signify efficacy. The efficacy of EUS CPN diminished at 8–12 weeks, after which pain scores in patients not receiving adjuvant therapy trended upward. While this study offers preliminary data suggesting the efficacy and safety of EUS CPN, the small sample size, absence of a placebo control group, and no physician or patient blinding limit the strength of the conclusions. These data considered in isolation do not allow us to make definitive conclusions regarding the safety and efficacy of EUS CPN in pancreatic cancer (Fig. 10).

Despite shortcomings in the literature, a review of existing data reached the following conclusions:

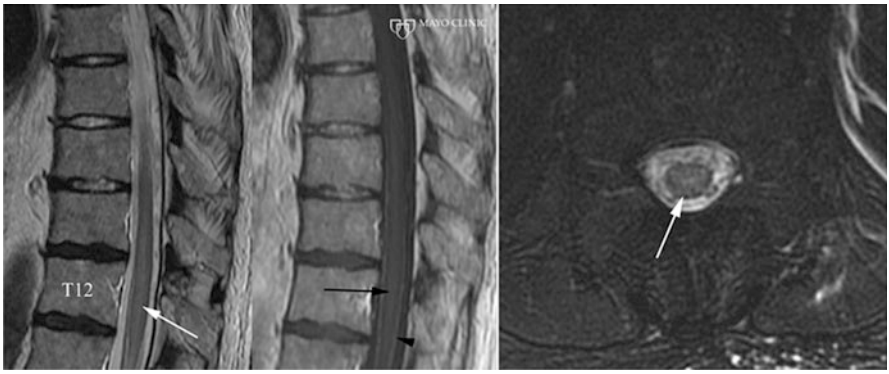

Fig. 10 Magnetic resonance imaging reveals decreased perfusion and an anterior spinal cord infarct following EUS-guided celiac neurolysis for pain management in a patient with unresectable pancreatic adenocarcinoma

1. The efficacy of CPN is similar regardless of the technique (PQ vs. EUS). This view is supported by the finding of a meta-analysis, which concluded that the efficacy of CPN was independent of the PQ approach or the use of radiologic guidance [75]. The reported efficacy rates of EUS CPN have been similar to those reported for PQ methods. Although comparative studies have not been performed, the efficacy is similar.
2. The risk of EUS CPN is similar or slightly lower than PQ methods. Many complications (such as paraplegia) have never been reported with EUS. This is likely because PQ methods are used far more often than EUS, as much as because of any difference in the inherent risk for a particular procedure. However, EUS is an "anterior" approach and thereby avoids the retrocrural space and may reduce this risk of neurologic dysfunction and pulmonary complications. Furthermore, as opposed to the PQ anterior approach, with EUS the needle only traverses the gastric wall, presumably eliminating complications resulting from inadvertent penetration of surrounding organs. The authors theorized that the risk of local pain, hypotension, diarrhea, and abscess formation would be similar for EUS and PQ approaches.

If EUS guidance offers no advantage in terms of pain relief, and no to minimal risk advantage, then one may wonder the role of EUS versus PQ techniques. The major disadvantage with EUS CPN is the inherent cost associated with the endoscopy and conscious sedation. However, the ability to perform EUS CPN at the time of tumor biopsy and staging combines diagnostic and therapeutic modalities which simplify patient care and may reduce cost. Most reserve EUS CPN for patients undergoing EUS for another reason, such as diagnosis or staging, for poor operative candidates, or those in whom disease spreads, precludes a satisfactory PQ approach.

The timing of the block relative to the onset of pain may predict response. In one study, CPN was more effective when performed early after pain onset rather than late in its course [66]. This may be explained by the fact that early pancreatic cancer pain appears to derive mainly from the celiac plexus. While most studies have found that CPN reduces cancer pain, it rarely eliminates pain and nearly all patients require continued opioid use, albeit often at a lower dose. When counseling patients, it is important to emphasize a realistic goal, which is not to eliminate pain, but to optimize oral pharmacologic therapy and to allow a dose reduction in order to minimize the side effects.

Based on an established classification system (Table 2) and definitions for level of evidence, the authors considered pancreatic cancer pain a Class IIa indication, as the weight of evidence favors the efficacy of EUS CPN [85].

Endoscopic Ultrasound-Guided Celiac Ganglia Neurolysis

Unfortunately, while CPN and CPB are considered safe, they provide limited benefit in terms of degree and duration of pain relief. The limited efficacy may partially be explained by the fact that until recently it was believed that the celiac ganglia could

Table 2 Classification system pertaining to the usefulness or efficacy of a certain procedure or treatment and associated level of evidence

Classification system
Class I: Conditions for which there is evidence or general agreement that a given procedure or treatment is useful and effective
Class II: Conditions for which there is conflicting evidence or a divergence of opinion about the usefulness/efficacy of a procedure or treatment
Class IIa: Weight of evidence/opinion is in favor of usefulness/efficacy
Class IIb: Usefulness/efficacy is less well established by evidence/opinion
Class III: Conditions for which there is evidence and/or general agreement that the procedure/treatment is not useful/effective and in some cases may be harmful
Level of evidence
Level of evidence A: Data derived from multiple randomized clinical trials
Level of evidence B: Data derived from a single randomized trial or nonrandomized studies
Level of evidence C: Consensus opinions of experts

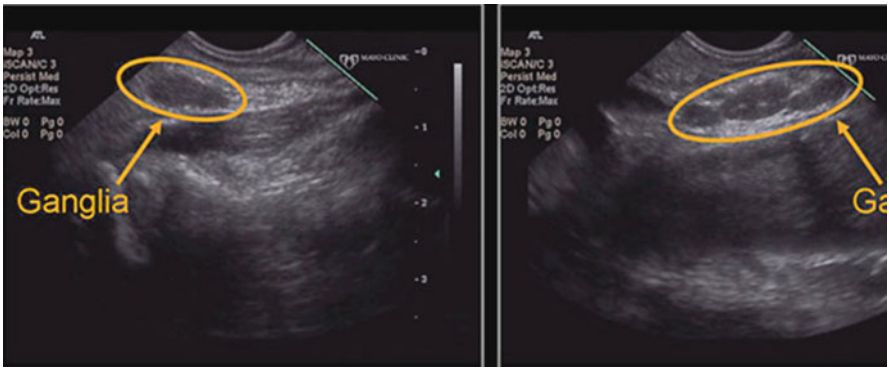

Fig. 11 Linear EUS images of celiac ganglia revealing hypoechoic oval or almond-shaped structures with irregular margins. Central echo-rich strands or foci may be present, and echo-poor threads are usually seen arising from ganglia

not be imaged. Therefore, with standard EUS, intraoperative, and anterior transcutaneous approaches, a needle was inserted adjacent and anterior to the lateral aspect of the aorta at the level of the celiac trunk in an attempt to deliver the injectate into the general region of the celiac plexus. The recent recognition that celiac ganglia can be visualized and accessed by EUS allows for direct injection into individual celiac ganglia to perform celiac ganglia neurolysis (CGN) and celiac ganglia block (CGB) (Figs. 11, 12, and 13). This more precise delivery of therapy offers the potential for enhanced efficacy and safety. To evaluate this hypothesis, a pilot study was conducted in patients with moderate to severe pain undergoing direct CGN for unresectable pancreatic carcinoma [86].

Eighteen patients underwent direct ganglia injection with bupivacaine (0.25%) and alcohol (99%) (Fig. 14). Clinical, technical, safety, and efficacy data are presented in Table 3. The mean age was 66 years (standard deviation [SD], 13.4 years; range,

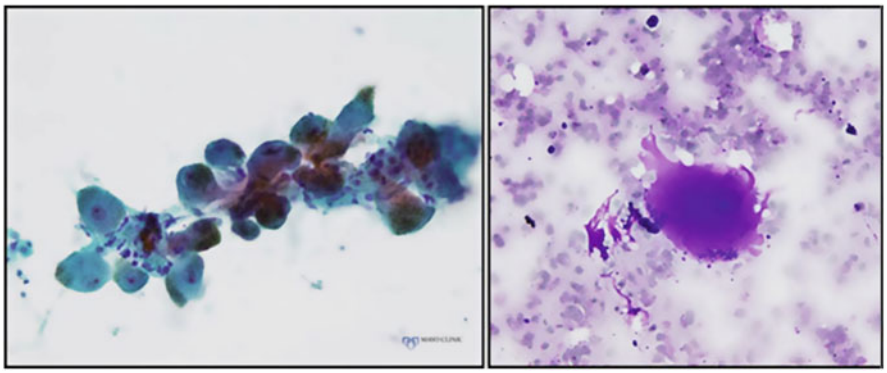

Fig. 12 Cytology specimens demonstrated nerve cell bodies without lymphocytes or malignant cells. The ganglion cells are large epithelioid cells with prominent nucleoli, with round-to-oval borders and abundant granular cytoplasm (*blue*/*purple*)

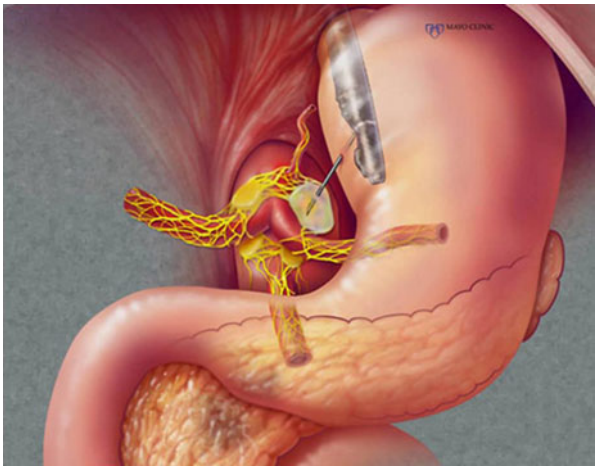

Fig. 13 Illustration demonstrates needle placement when performing direct celiac intra-ganglia neurolysis

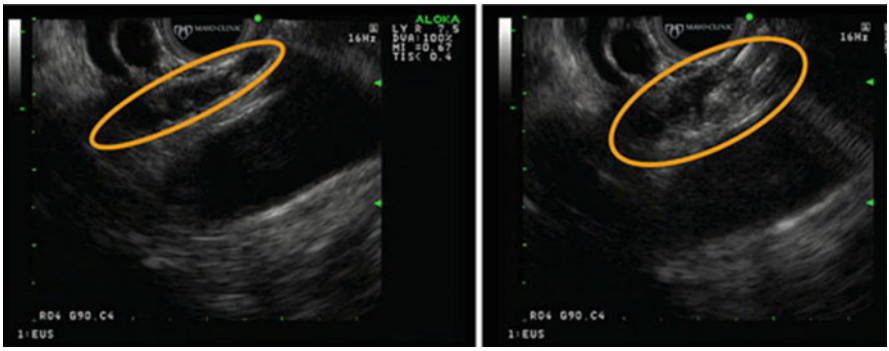

Fig. 14 Linear EUS images of celiac ganglia (*left* panel) and a corresponding direct celiac intra-ganglia injection (*right* panel)

Table 3 Clinical, technical, safety, and efficacy data following EUS CGN

Disease	Age (years)	Ganglia identified	Ganglia injected	Bupivacaine volume (ml)	Alcohol volume (ml)	Depo-medrol (mg)	Pain relief (complete or partial)
	Mean (range)						
Pancreatic cancer	66	3.0	2.7	8.3	12.7	80.0	Alcohol 16/17 (94%)
	(39–80)	(1–6)	(1–6)	(1–17)	(2–20)	(80, $n = 1$)	Steroid 0/117 (9)
($n = 18$)				($n = 18$)	($n = 17$)		$P = 0.004$

EUS endoscopic ultrasound, *CGN* celiac ganglia neurolysis, *CGB* celiac ganglia block

39–80 years), including eight males and ten females. Ganglia could be seen and accessed in 33 of 35 (95%) of patients. A mean of 3.0 (range 1–6) ganglia was identified, and a mean of 2.7 (range 1–6) ganglia was injected. Patients reported pain relief in 16/17 (94%) when alcohol was injected and 0/1 (00%; $p = 0.004$) when steroid was injected. For those who reported pain relief, 16 patients reported partial relief and none experienced complete pain relief. Narcotic use increased in 2 patients, remained equivalent in 13 patients, and decreased in 3 patients.

Patients were described as having "immediate" pain when discomfort was observed, while the needle was within the ganglia despite sedation. This pain was distinguished from "initial" pain exacerbation, which began in the recovery room or soon thereafter. Seven patients experienced an "initial" pain exacerbation lasting a mean duration of 2.2 days and requiring hospitalization in one patient. Notably, patients who developed an initial pain exacerbation tended to eventually experience greater pain relief at follow-up. All seven patients (100%) who had an initial pain exacerbation reported eventual efficacy of CGN versus 9 of 11 (81%; $p = 0.23$).

Transient hypotension defined by a decrease in blood pressure exceeding 20 mmHg systolic or 10 mm Hg diastolic occurring within 3 min of upright tilt developed in six (33%) patients, one of whom required additional fluid administration. Four (22%) patients subjectively noted marked ($n = 2$) and mild ($n = 2$) improvement of their narcotic-induced constipation. There was no evidence of other complications and specifically no patients described any neurologic deficits. The retrospective, noncontrolled nature of the study, which included a limited enrollment, provided inadequate power to permit firm conclusions and raises as many questions as it answers.

Methodological limitations include the varied and noncontrolled technique for injection, composition of the injectate, use of general descriptors of pain response instead of a visual analog scale, lack of a precise measure of the impact on opioid analgesic consumption, brief duration of follow-up, and lack of correlation with quality of life and with survival. Recognizing these limitations, the objective was simply to provide pilot data regarding the safety and initial efficacy of direct CGN and CGB in patients with moderate to severe pain resulting from unresectable

pancreatic carcinoma or chronic pancreatitis. The varied study limitations prohibit one from making firm conclusions regarding the safety or efficacy of this modified approach to pain management. However, initial data suggest that in patients with moderate to severe pain secondary to pancreatic cancer, the direct CGN with alcohol injection is safe and effective in initial pain management. Interestingly, a subsequent review of patients with unresectable PC who underwent neurolysis, either celiac plexus or celiac ganglia over a 12-year period, noted that neurolysis was an independent predictor of shortened survival [87]. Prospective, controlled, and comparative trials are needed to confirm the safety and assess the long-term efficacy of the ganglia neurolysis approach to pain management relative to conventional techniques.

Locally Injected Antitumor Therapies

Almost 40 years ago, it was shown that combined 5-fluorouracil (5-FU) and radiation therapy prolonged median survival to 9 or 10 months for patients with locally advanced pancreatic cancer (LAPC) [88]. Gemcitabine and FOLFIRINOX are now the standard chemotherapeutic agents for LAPC following evidence to suggest superior results to 5-FU [89–91]. Such agents act as a radiosensitizer through nucleotide pool alterations, cell cycle redistribution, induction of apoptosis, inhibition of DNA synthesis, and altered DNA repair [92–94]. Despite evaluation of more than 30 new agents designed to enhance the effect of chemoradiotherapy, there has been little advance with each drug, failing to produce meaningful improvement in the resectability rate or survival. FOLFIRINOX is associated with a survival advantage but with an increased toxicity profile. Indeed, currently the median survival of patients with LAPC is only 3–6 months with a 5-year survival of a dismal 7% [95].

Conventional multimodality therapy is minimally effective in patients with LAPC and even less so when attempting to downstage tumors to allow R0 resection. The poor efficacy results not only from the tumor biology but likely also because of dose limitations necessary to limited damage to normal tissues. Efficacy is further limited by current problems with drug delivery that may be overcome by direct IR or EUS-guided intratumoral injection. Their use may allow increased intratumoral drug concentrations and augment the efficacy of chemoradiation while minimizing the risk. Although speculative, locally directed therapies are likely to target the primary tumor with minimal impact on likely sites of local infiltration and distant metastasis. There is need to develop these techniques and chemotherapeutic agents to allow spread of the injected agents along the same patterns of metastasis as assumed by the cancer itself. Patients may also benefit and require use of other locally delivered ablative therapies (discussed later in this chapter) as well as concomitant systemic therapies. Experience and data are limited at this time and generally regarded as investigational, but will be briefly reviewed herein.

Interventional Radiology (IR)-Guided Injection

Intra-arterial Injection

The pancreaticoduodenal arcade is the targeted arterial system of interest for pancreatic head pathology. Branches of the superior mesenteric artery (SMA) and the transverse pancreatic artery are important landmarks to gain access to targeted areas of the pancreatic body and tail. The pancreatic arteries are selectively cannulated and a catheter is placed into the vessel of choice to allow chemotherapeutic embolization.

In a pilot study, 20 patients with unresectable pancreatic cancer underwent continuous 5-FU infusion for 5 days a week for 5 weeks, with concurrent radiation therapy [96]. A partial response was seen in 70% of patients. The 1- and 3-year survival rates were 40 and 17%, respectively, with median survival duration of 11 months. A similar study, which also included systemic gemcitabine therapy, demonstrated a partial response rate of 21% [97]. A phase I trial involving arterial infusion of gemcitabine and 5-FU resulted in an overall response rate of 33% with 1- and 2-year overall survival occurring in 83 and 25% of patients, respectively [98]. Other agents have been used to include cisplatin [99].

Others have targeted therapy into larger caliber vessels including the celiac trunk via the femoral artery, as was true in one study of 211 patients injecting the FLEC regime (5-fluorouracil, leucovorin, epirubicin, and carboplatin) once every 3 weeks for a combined total of 764 cycles, approximating 3 cycles per patient [100]. Prognostic factors of overall survival in patients receiving this particular regimen included pain reduction, disease stage, and the number of administered intra-arterial chemotherapy cycles.

A separate catheter may be inserted to allow hepatic infusion to potentially prevent or treat liver metastasis. This method is primarily reported for management of hepatic metastases secondary to colorectal cancer. Hepatic arterial infusion of 5-FU in patients with liver metastasis specifically from pancreatic carcinoma has also been reported but was considered to be minimally effective using a 5-day regime by continuous hepatic arterial infusion every 4 weeks [101]. Alternatively, established hepatic metastasis may be treated by a weekly hepatic arterial infusion of 5-FU in addition to external beam radiation therapy (total dose, 50 Gy; 2 Gy day^{-1}). Following a median of 13 cycles of chemotherapy, a partial response of 41% with a 1-year overall survival of 11.8% was observed [102].

Computed Tomography Injection

Oncolytic viruses for the treatment of pancreatic cancer studied in recent experimental and clinical work include adenoviruses, herpesviruses, and reoviruses. These replication-selective viral agents hold promise as a novel cancer treatment platform (virotherapy). ONYX-015, an E1B-55 kDa gene-deleted adenovirus, was the first such genetically engineered agent to be tested in humans. In combination with chemotherapy, some antitumoral activity has been demonstrated. CT-guided injection of ONYX-015 which is thought to preferentially replicate within and kills malignant

cells has been evaluated [103]. Although well tolerated, the results showed no objective tumor response and no viral replication. Unfortunately, as tumor seeding is a well-recognized complication of any CT-guided percutaneous intervention in cancer patients, this may not be the most suitable method of direct intratumoral administration [104]. Therefore, alternative and improved delivery methods are required for the local injection therapy to manage patients with pancreatic cancer.

Endoscopic Ultrasound (EUS)-Guided Injection

EUS-guided FNI is a relatively new method system for the delivery of antitumor agents. The initial fine needle aspiration (FNA) of a pancreatic malignancy was reported in 1992 and is now a routine part of EUS examination [105]. The initial indications for FNA were proposed by Erickson and have been modified over time to include (1) sampling of pancreatic masses when other techniques have failed, (2) sampling CT-detected mediastinal adenopathy when other techniques have failed, (3) distinguishing benign from malignant disease, and (4) staging of cancer to provide evidence of malignancy prior to neoadjuvant therapy or to guide palliative care [106]. The pancreas and biliary tree are generally observed from three regions: the stomach, the duodenal bulb, and the second portion of the duodenum. The identified areas are accessible for fine needle aspiration and, by default, amenable to fine needle injection. The echoendoscope and current needle devices are the same as that used for standard FNA. This is an emerging indication for EUS, but merits some mention to highlight the potential role, while awaiting further studies accompanied by technical success, clinical success, and adverse event data.

As with EUS FNA, FNI is optimal when the target lesion is visualized but merits some mention to highlight the potential role, while awaiting further study of fine needle aspiration and, by default, amenable to fine needle injection, but injections per session, volume and composition of the injectate, and total number of sessions vary among reports. The only direct injection of standard gemcitabine chemotherapy for patients with locally advanced or metastatic pancreatic cancer has been reported by Levy et al. highlighting the safety and feasibility in 36 patients [107] (Fig. 15).

Allogenic Mixed Lymphocyte Culture (Cytoimplant)

EUS FNI was introduced as a new means for local delivery of antitumor agents for patients with locally advanced pancreatic carcinoma (LAPC) in a study published in 2000. In a phase I clinical trial, eight patients underwent EUS-guided administration of an allogenic mixed lymphocyte culture (cytoimplant) to treat patients with LAPC (four patients in Stage II, three in Stage III, and one in Stage IV) [108]. Cytoimplants were delivered locally into the tumor using a novel EUS-guided FNI technique. Escalating doses of three, six, or nine billion cells were implanted into the pancreatic tumor by a single EUS-guided FNI. Toxicity (modified National Cancer Institute criteria) was assessed at day 1, week 1, and months 1 and 3, whereby there were no bone marrow, hemorrhagic, infectious, renal, cardiac, or pulmonary toxicities.

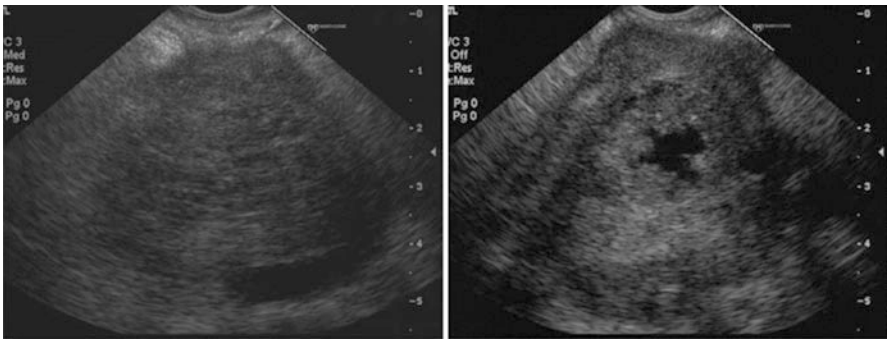

Fig. 15 EUS imaging demonstrates a pancreatic cancer prior to therapy (*left*) and following fine needle injection of gemcitabine with the resulting effects demonstrating and altering echodensity and echo pattern (*right*)

However, a low-grade fever was experienced by 86% and responded to acetaminophen, resolving within 28 days. There were no reported procedure-related complications suggesting that a single injection of cytoimplant immunotherapy by EUS-guided FNI may be safe.

ONYX-015

Oncolytic virus therapy was developed early in the last century upon observing occasional tumor regression in cancer patients suffering from viral infections or those receiving vaccinations. EUS FNI has also been applied to the delivery of the antitumor agent ONYX-015 (Onyx Pharmaceuticals, USA), which is a gene-deleted replication-selective adenovirus that preferentially kills malignant cells. In a phase I/II clinical trial designed to demonstrate feasibility, tolerability, and efficacy, 21 patients underwent eight injections over an 8-week period [109]. The FNI protocol consisted of 1 ml of virus per injection, with up to ten injections per session depending on tumor size. The latter four treatments were given in combination with gemcitabine on the same day. Four complications were encountered including sepsis ($n = 2$) and duodenal perforation ($n = 2$). Eleven patients had progressive disease or evidence of treatment toxicity. No clinical pancreatitis occurred despite mild, transient elevations in serum lipase levels in a few patients. Two patients had partial regression, 2 patients had a minor response, and 11 patients had disease progression or toxicity prohibiting study completion. Phase I clinical trials are underway using alternative oncolytic viral therapies including herpes virus hrR3 and HSV-1 strain HF clone, which are specifically designed to express other genes to increase the susceptibility of tumor cells to chemotherapy. The results are awaited and may provide future potential EUS FNI antitumoral agents.

TNFerade™

More recently EUS has been used to deliver TNFerade™ antitumor therapy in a dose-escalating study and to obtain initial data regarding safety [110, 111]. TNFerade™ is a second-generation adenovector, which expresses the cDNA encoding human tumor necrosis factor (TNF). In addition, a radiation-inducible immediate response *Egr-1*

(early growth response) promoter was placed upstream of the transcriptional start site of the human TNF. In a pilot study involving 50 patients, TNFerade was administered either percutaneously via ultrasound or CT, or via EUS guidance. Local therapy was given in conjunction with continuous systemic intravenous 5-FU [112]. TNFerade was injected by a single percutaneous needle pass or up to four EUS injections. Dose-limiting toxicities developed in 4 of 50 patients including pancreatitis in two patients and hypotension and biliary obstruction in one patient each. No severe complications were noted. At the maximum tolerated dose (MTD), four out of five patients reassessed as surgically resectable achieved pathologically negative margins, and three survived 24 months. Intratumoral therapy did not interfere with subsequent surgical resection.

OncoGel (ReGel/Paclitaxel)

OncoGel (ReGel/paclitaxel) is a new formulation for intralesional injection of the chemotherapeutic drug paclitaxel, developed by MacroMed Inc. (Sandy, Utah), for local tumor management. OncoGel uses MacroMed's ReGel drug delivery system, a thermosensitive, biodegradable triblock copolymer composed of poly(lactide-co-glycolide)-polyethylene glycol-poly(lactide-co-glycolide). Upon injection and in response to body temperature, ReGel is transformed from a water-soluble polymer to a water-insoluble biodegradable hydrogel that releases paclitaxel continuously into the adjacent tissue for up to 6 weeks [113]. In a porcine pancreas study, EUS FNI with OncoGel demonstrated high and sustained localized concentrations of paclitaxel highlighting a further potential minimally invasive local treatment option for unresectable pancreatic tumors [114, 115]. In a canine model, another polymer implantation alternative for interstitial chemotherapy has demonstrated successful implantation and localized tissue necrosis in the absence of significant complications [116]. Clinical data are unavailable.

Immature Dendritic Cells

Dendritic cells are potent antigen-presenting cells utilized to induce primary T-cell dependent immune responses. When the cells are injected intratumorally, they acquire and process tumor antigens in situ and migrate to regional lymph nodes whereby they initiate a strong tumor-specific immune response. A pilot study investigated the feasibility, safety, and clinical response following EUS FNI of immature dendritic cells into pancreatic cancer deemed refractory to systemic gemcitabine therapy [117]. The study included seven patients who received EUS FNI intratumoral injection of immature dendritic cells at two to three sites. Five of seven patients received radiation therapy before initial EUS FNI of dendritic cells to induce apoptosis and necrosis. There were no reported complications and the median survival period was 10 months.

Ablative Antitumor Therapies

A variety of imaging techniques have been used to guide pancreatic tumor ablation including ultrasound (percutaneous, intraoperative, and endoscopic), CT, CT fluoroscopy, MRI, and fluoroscopy. Available ablative therapies include brachytherapy,

cryoablation, radiofrequency ablation (RFA), microwave coagulation therapy (MCT), laser interstitial thermal therapy, and high-intensity focused ultrasound (HIFU). However, only few are utilized in the management of pancreatic cancer. The following discussion will focus predominantly on EUS-guided methods for delivering ablative therapy. Experience and data are limited at this time and generally regarded as investigational or preliminary human experience, but will be briefly reviewed herein.

Brachytherapy

Traditional Brachytherapy

Prostate brachytherapy, with transrectal ultrasound-guided placement of radioactive seeds, is an effective treatment option for early-stage prostate cancer [118, 119]. Its potential advantage over traditional external beam radiation therapy is the ability to limit radiation toxicity to the surrounding normal tissues. Following radioactive seed placement, the target tissue is exposed to gamma rays, which in turn produce localized tissue injury and tumor ablation.

Brachytherapy (iodine, gold, iridium) delivered by percutaneous approaches to pancreatic tumors has not been particularly effective. Percutaneous intratumoral injection with radioactive 32P has also been reported, but with disappointing results [120–122]. Memorial Sloan Kettering Cancer Center evaluated their initial experience in 98 patients with biopsy-proven unresectable pancreatic adenocarcinoma from 1974 to 1987 [123]. Patients were treated with I-125 implants during laparotomy performed for biopsy alone, gastric bypass, biliary bypass, and partial or total pancreatectomy with incomplete resection. In addition, 27 patients received postoperative external irradiation and 27 patients received chemotherapy. Postoperative complications included postoperative death, biliary fistulae, intra-abdominal abscess, GI bleeding, gastric or small bowel obstruction, sepsis, and deep vein thrombophlebitis. A multivariate analysis highlighted that four factors significantly affected survival: (1) T stage, (2) N stage, (3) administration of chemotherapy, and (4) >30% reduction in the size of the implant on follow-up films. A subgroup of patients with T1N0 stage disease who received chemotherapy survived 18.5 months. A percutaneous ultrasound study of 19 patients reported no difference in survival or palliation between patients treated with I-125 seed implantation compared with those treated with seeds and external radiation despite satisfactory seed placement and delivery of the planned radiation dose in most cases [124].

Computed tomography was used to guide therapy in 26 patients who had a mean tumor size of 6.1 cm by inserting I-125 seeds at a distance of 1.0–1.5 cm [125]. Over the 3–12 months of follow-up, complete symptom relief was seen in nine patients, partial relief in two, and no change in four with an effective rate of 73%. A CT performed 2 months following implantation demonstrated complete response, partial response, no change, and progression in 2, 13, 5, and 5 cases, respectively, with an

overall effective rate of 57.7%. Migration of the seeds into the liver was seen in three patients. No severe complications were reported.

In another pilot study, intraoperative or percutaneous cryosurgery was performed under ultrasound and/or CT guidance, and the less invasive form of therapy was found to result in a lower rate of adverse events [126]. I-125 seed implantation can destroy residual surviving cancer cells following cryosurgery. Hence, a combination of modalities may augment the effects, both beneficial and potential detrimental. Others believe that brachytherapy, when combined with external radiation therapy and systemic chemotherapy, probably provides the best local control of pancreatic cancer, but these contentions cannot be validated based on current data [127].

Placement of fiducials within pancreatic cancer tumor enables easy identification of the target lesion during radiation therapy. Therapy can be delivered in a precise and targeted manner despite respiration excursion. Although percutaneous placement of fiducials is possible using CT guidance, the procedure is technically cumbersome and there is concern regarding tumor seeding [128, 129]. To date, reports regarding CT-guided placement of fiducials pertain almost entirely to the therapy of lung cancer.

EUS-Guided Delivery of Fiducial Markers and Brachytherapy

EUS-guided fiducial marker placement has been reported in patients with mediastinal or intra-abdominal tumors including pancreatic carcinoma in patients scheduled to undergo stereotactic radiosurgery [130–133]. A total of three to six fiducials were placed in each patient. The impact of EUS-guided fiducial placement and stereotactic therapy on patient survival or quality of life is unknown.

EUS-guided implantation of radioactive seeds into pancreatic parenchyma of a porcine model ($n = 6$) was reported to be a safe, simple, and minimally invasive technique for interstitial brachytherapy [134]. The radioactive iodine seeds were inserted into the lumen of the tip of a modified EUS needle, which had a normal 22-gauge needle body with a 2.5-cm-long, 18-gauge needle tip attached to the distal end. In a pilot trial of 15 patients with advanced pancreatic cancer, EUS-guided interstitial brachytherapy was evaluated with respect to tumor response, clinical response, safety, and complications [135]. A mean number of 22 radioactive seeds per patient were implanted into the tumors. It had a moderate local tumor effect and a clinical benefit was demonstrated in 30%. Complications were experienced by six patients to include pancreatitis, pseudocyst formation, and hematologic toxicity. Another study involving 22 patients, which successfully implanted a median of ten radioactive iodine seeds per patient, suggested an improvement in pain scores the first week post procedure, which however was not sustained, and no long-term survival benefit was demonstrated [136].

The future of this particular ablative method has yet to be established primarily because of the uncertain impact on patient outcomes and due to issues regarding how best to handle radioactive material in the endoscopy suite and proper disposal of radioactive accessories.

Photodynamic Therapy

Traditional Photodynamic Therapy

Photodynamic therapy works through the induction of apoptosis and tissue necrosis caused by a direct cellular action and/or by altering tumor blood supply. In the mid-1970s, the feasibility of tumor eradication with photodynamic therapy (PDT) was demonstrated in animal models, and the first patient studies were reported shortly thereafter [137]. Studies have subsequently revealed that PDT produces local tissue necrosis, and in experimental studies, it has been shown to deliver relatively tumor-specific injury with minimal injurious effect to the normal surrounding pancreatic and peripancreatic tissues [138–140]. A randomized controlled trial of PDT with 5-aminolevulinic acid for implanted pancreatic cancers in hamsters highlighted that survival time in the group treated with PDT was significantly greater than that of control animals [141].

EUS-Guided Photodynamic Therapy

EUS-guided PDT of the pancreas was initially studied in a porcine model [142]. Following injection of porfimer sodium, a 19-gauge needle was inserted into the pancreas, in addition to other organs under EUS guidance. Subsequently, a small-diameter quartz optical fiber was passed through the EUS needle and used to illuminate the tissue with laser light. Localized tissue necrosis was achieved without significant complication. To date, no comparative study has been performed between PDT and external beam radiation. An alternative photosensitizer (verteporfin) has also been evaluated and was associated with less photosensitivity than porfimer sodium, while achieving localized pancreatic tissue ablation of porcine pancreas in a dose-related fashion [143]. Preliminary data from four patients with advanced pancreaticobiliary disease suggests that EUS-guided PDT with a second-generation photosensitizer (chlorin e6 derivative) and a flexible laser probe is feasible and safe [144].

Radiofrequency Ablation

Traditional Radiofrequency Ablation

Image-guided percutaneous radiofrequency ablation (RFA) has been increasingly performed in recent years to treat solid tumors. Radiofrequency ablation renders a zone of coagulation necrosis by an intense thermal burn. It has been used as an ablative modality in the setting of primary and secondary liver lesions either surgically (laparoscopically or open) or percutaneously by ultrasound-, MRI-, or CT-guided methods. Cryotherapy and radiofrequency treatment can ablate metastases in 50–90% of cases and are relatively safe compared to hepatic resection. The goal of RF thermal ablation is to destroy the tumor as well as a 5 ± 10-mm circumferential cuff of adjacent normal hepatic parenchyma. There has been no randomized comparison using RFA in the setting of primary pancreatic cancer or associated hepatic metastases. A recent study of 18 patients with unresectable

disease, treated by RFA with a "cool-tip needle," highlighted that the most notable effect was in relieving back pain and was safer if used in the pancreatic body/tail region [145]. Smaller studies have demonstrated similar results highlighting that to date RFA of unresectable pancreatic carcinoma is feasible, efficacious, and safe and is also feasible for metastatic lesions to the pancreas to include renal cell carcinoma [146–148]. Initial clinical experience has shown that approximately one-third of patients develop low-grade fever and flu-like symptoms, which include malaise, myalgia, and nausea and/or vomiting, after RFA [149].

An ex vivo model of RFA of the porcine pancreas was evaluated to determine the thermal kinetic profile of the ablation effect as there have been some concerns regarding injury to the duodenum, bile duct, or portal vein [150–152]. In practice, small tumors (less than 3 cm) are more likely to be resected, and thus ablation will potentially see clinical use in larger unresectable pancreatic tumors, where a more prolonged ablation time may be required. A minimal duration of 5 min is probably required to produce a 2 cm area of ablation [150].

EUS-Guided Radiofrequency Ablation

The feasibility and effectiveness of RFA under EUS guidance in the porcine pancreatic tail has been confirmed by necroscopy [153]. Radiofrequency was applied for 6 min with a goal tip temperature of 90 °F and the subsequent area of necrosis measured 1 cm. Biochemical parameters were normal in all except in one pig that developed pancreatitis and an associated pancreatic fluid collection. Other complications included gastric and intestinal burns secondary to misplacement of electrodes. The simultaneous combination of RF and cryoablation with the use of a novel applicator design yields significantly larger coagulation zones than either modality alone [154]. Since the initial porcine studies, its role, although preliminary in nature, has been reported for the management of cystic neoplasia, insulinomas, and unresectable pancreatic cancer [155–157]. Reported adverse events related to EUS RFA have included acute pancreatitis, gastric wall burns, and gut adhesions [158].

EUS-Guided Alcohol Ablation Therapy

Until a few years ago, tumor ablation therapy consisted of the injection of sclerosing agents (i.e., absolute alcohol) into primary or metastatic tumors of the liver. Percutaneous ethanol injection (PEI) of large and multiple hepatocellular carcinomas showed survival similar to conventional PEI for patients with smaller tumors [159]. Although alcohol ablation therapy has been a successful mode of therapy, its use has generally been confined to patients with cirrhosis whose tumors are anatomically amenable to a percutaneous approach.

EUS-guided alcohol injection of solitary hepatic metastasis and adrenal metastases and ablation of gastric stromal cell submucosal and pancreatic neuroendocrine tumors have been reported [160–163]. This alternative ablative therapy with an ethanol injection was performed in a pilot study to determine if pancreatic tissue

ablation (98% ethanol preparation) could be safely performed and to attempt to define the dose response of pancreatic tissue to ethanol [164]. All animals demonstrated some degree of fibrosis and necrosis, with no significant difference between 98% and 50% ethanol. A subsequent study used 2 ml of ethanol, in an increasing concentration of 0–100%, and determined that the cross-sectional area of necrosis was proportional to the concentration of ethanol [165]. The use of contrast-enhanced EUS with microspheres improves visualization of altered pancreatic vascular perfusion and can be used to facilitate detection of small pancreatic lesions and respective follow-up after ablative therapy [166]. Ethanol has also been used to ablate a functioning insulinoma in a nonsurgical candidate [167, 168].

Conclusion

Interventional radiology and EUS are performed in patients with pancreatic cancer to allow diagnosis, staging, and increasingly now with therapeutic intent. In the setting of malignant biliary and gastric outlet obstruction, there are established standards of care derived from evidence-based guidelines. There are a variety of modalities to ameliorate the pain associated with pancreatic cancer, and direct intraganglionic therapy via EUS is a realistic potential route for therapy. However, further research is necessary to evaluate injection and ablative therapies, which are currently in the early stages of development and human study. These latter techniques cannot be widely advocated as a part of routine clinical care until their efficacy, technical success, and respective safety profiles have been established.

Key Research Points

- Interventional radiology and EUS-guided procedures are increasingly utilized in the care of patients with pancreatic carcinoma. As new devices and techniques are introduced and validated, they tend to replace an equivalent surgical procedure.
- Endoscopic palliative treatment of malignant biliary and duodenal stenosis using metal prostheses is highly feasible, safe, and effective including in patients with combined obstructions even with associated biliary stenting.
- With the advent of EUS-guided celiac plexus neurolysis, an alternative route to percutaneous access has been developed. As ganglia are now readily visualized by EUS, they may be a future target for fine needle injection therapies.
- EUS ablation of solid tumors and lymph nodes is primarily investigational although reports are beginning to emerge particularly in the field of RFA describing their clinical application.
- A variety of agents are available but seldom used at this time for tumor ablation including ethanol, gel-based and polymer-based chemotherapies, brachytherapy seeds, radiofrequency ablation, and attenuated viral vectors.

Published Guidelines None

- No specific published guidelines.

Future Research/Directions

In patients with malignant biliary and/or duodenal obstruction to:

- Develop larger, long-term, prospective randomized controlled trials that evaluate cost, clinical outcomes, and quality of life following biliary and/or duodenal bypass.
- Identify factors that reliably predict survival and stent patency. This information is crucial in identifying patients who will most benefit from initial placement of an expandable SEMS as opposed to those who may be well palliated with a less expensive plastic stent.
- Develop new, or improve existing, technologies to prolong stent patency.
- Develop the techniques and accessories (catheters and guidewires) to improve upon the novel endoscopic approaches for bypassing malignant biliary obstruction.

In patients with pancreatic carcinoma related pain to:

- Develop larger, long-term, prospective randomized controlled trials that evaluate cost, clinical outcomes, and quality of life following celiac plexus neurolysis (CPN) and/or celiac ganglia neurolysis (CGN). Only then can the verification of the efficacy and safety of EUS CPN and identify advantages and disadvantages of the various techniques.
- Determine the (1) optimal timing and route for CPN/CGN, (2) ideal composition of the injectate, (3) cost, (4) patient preference, (5) influence on quality of life, (6) effect of neurolysis (duration in those with chronic pancreatitis), and (7) potential survival advantage.
- With regard to local tumor therapies to:
  - Develop larger, long-term, prospective randomized controlled trials that evaluate cost, clinical outcomes, and quality of life following these therapies.
  - Develop 3-D mapping systems or devices to more precisely control and deliver the dosing of ablative energy.
  - Determine if a combination of local with or without systemic ablative therapies may provide a synergistic affect.

Cross-References

► MRI and MRCP for Diagnosis and Staging of Pancreatic Cancer
► Therapeutic Endoscopy in the Management of Pancreatic Cancer

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Palliative Surgery in Advanced Pancreatic Cancer

Florian Scheufele and Helmut Friess

Contents

Abstract

In patients with pancreatic cancer, a high percentage is not eligible for curative treatment, and therefore palliative care is indicated. Malignant obstructive jaundice, severe pain, and gastric outlet obstruction (GOO) contribute a major compromise to patients' quality of life. To manage these symptoms, different strategies of treatment, either surgical or interventional, are available.

Obstructive jaundice can either be treated by hepatico-/choledochojejunostomy or by interventional placement of a biliary stent. Patency of surgical bypasses by hepaticojejunostomy is longer, when compared to interventionally placed biliary stents. However, self-expandable metal stents (SEMS) display better patency rates, expanding the spectrum of biliary stenting also on patients with longer life expectancy.

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GOO significantly contributes to decreased quality of life (QOL). Patients with an unresectable pancreatic cancer at exploration should receive a gastrojejunostomy. Surgical palliation for GOO should be considered due to superiority to interventional duodenal stenting if life expectancy is longer than 2 months.

Pain can sufficiently be treated with neurolytic interventions. Splanchnicectomy provides more sufficient pain relief than neurolysis of the celiac plexus. Prior to neurolytic interventions, a sufficient pharmacological analgesic treatment should be undertaken, and neurolysis should be performed early during workup if irresectability is evident.

In light of potent neoadjuvant treatment regimens, today's resection polices are getting more aggressive, and exploration should be performed whenever possible. If irresectability is evident during operation, R2 resections should be avoided due to increased postoperative complications and no survival benefit. In these situations, a double bypass surgery is always a better option.

Keywords

Malignant obstructive jaundice · Advanced pancreatic cancer · Surgical bypass · Interventional biliary drainage · Hepaticojejunostomy · Surgical palliation · Self-expandable metal stents · Gastric outlet obstruction · Gastrojejunostomy · Pain · Neurolysis · Splanchnicectomy · Palliative pancreaticoduodenectomy · R2 resection · Exploration

Introduction

Pancreatic cancer is a major cause of cancer-related death, currently lying behind lung cancer, prostate cancer, breast cancer, and colorectal cancer and conferring 7% of cancer-related deaths in the USA. For the year 2016, there are 53,070 new cases of pancreatic cancer estimated for the USA with a total number of 41,780 estimated new deaths during the course of this year. Although 5-year survival is still poor lying around 8% (2005–2011), advantages in treatment of pancreatic cancer are visible, as there has been a significant improvement of the 5-year survival rate having been 3% 30 years ago ($p < 0.05$) [1]. To date, consensus exists that resection of pancreatic cancer is the only chance for cure, although even after radical resection survival remains limited, and resection often ends in a palliative situation [2]. Median survival after curative resection with adjuvant chemotherapy has been reported with 23 months (using fluorouracil plus folinic acid) and 23.6 months (using gemcitabine), respectively, in the ESPAC-3 trial [3]. On the other hand, patients receiving palliative treatment have worse prognosis, although even in unresectable disease progress has been made using the FOLFIRINOX treatment regime reaching a median survival of approximately 11.1 months [4]. Further emphasizing the importance of palliative treatment for patients with pancreatic cancer is the low number of resectable tumors at the time of diagnosis with only approx. 1/3, when compared to 1/3 of the patients with locally advanced tumors at primary diagnosis

and 1/3 with metastatic disease leading to a 1-year survival of 74% (resection), 30% (chemotherapy/chemoradiation), and 16% (chemotherapy/chemoradiation) in the respective groups [5]. Thus, palliative treatment of patients suffering from pancreatic cancer is a major field facing common symptoms of advanced pancreatic cancer as obstructive jaundice, malignant gastric outlet obstruction, and severe pain. These symptoms are significantly compromising patient's quality of life, and amelioration can either be achieved by surgical or interventional treatment. The advantages and disadvantages of the different techniques are analyzed and outlined in the following.

Surgical and Interventional Treatment of Obstructive Jaundice

Malignant obstructive jaundice is a common symptom of advanced pancreatic cancer and tumors of the pancreatic head. If jaundice is untreated, it can give rise to consecutive diseases as cholangitis, progressive liver dysfunction, secondary liver cirrhosis, renal dysfunction, organ failure, and finally death. While obstructive jaundice due to benign diseases should be treated surgically to reach a definitive solution, in malignant disease, the picture is less clear related to the life expectancy of the patient. On the background of limited expected survival, morbidity and mortality associated with surgical treatment have to be critically evaluated, and interventional methods of drainage thereby have a significant value. On the other hand, the latter are associated with procedure-related complications such as stent migration, occlusion, and cholangitis, making readmission, replacement of the stent, and antibiotic therapy necessary. Thus, no final consensus exists on the ideal treatment strategy of those patients. Especially on the background of improved perioperative care, these different strategies for palliation of obstructive jaundice have to be reevaluated [6].

In the early 1990s, three controlled randomized clinical trials have been published comparing stenting of obstructive jaundice with surgical bypass [7–9]. These studies revealed that both procedures had high technical (93% vs. 95%) and clinical success rates (91% vs. 92%). Procedure-related mortality (3% vs. 14%, $p = 0.01$), major complications (11% vs. 29%, $p = 0.02$), and hospital stay (20 vs. 26 days, $p = 0.001$) were lower in the stented group. On the other hand, the incidence of recurrent jaundice (2.0% vs. 36%) and gastric outlet obstruction (7% vs. 17%) was reduced in the surgical group, when compared to stented patients. However, these studies are nowadays more than 20 years old, and in regard of improvements in stenting and in surgical techniques, e.g., minimal invasive surgery, new RCT should be initiated on this topic.

Besides endoscopic stenting, percutaneous transhepatic stenting offers an alternative method of decompression of the biliary tree. Transhepatic biliary stenting due to increased invasiveness and associated complications does not depict the first-line approach to decompress obstructive jaundice. Although after repeated unsuccessful endoscopic stenting or in case of impossibility of endoscopic stenting (e.g., after Billroth procedure), it has its benefits. Bornman and coworkers compared palliation of malignant obstructive jaundice by percutaneous transhepatic placement of a

biliary stent ($n = 25$) with surgical bypass ($n = 25$) in a randomized controlled trial with patients suffering from unresectable pancreatic cancer. Technical success was 84% in the stent and 76% in the surgical group, and postoperative complications (stenting 7 (28%) vs. surgery 8 (32%)) and 30-day mortality (stenting 2 (8%) vs. surgery 5 (20%)) were equal in both groups. Recurrent jaundice however was increased after percutaneous biliary decompression (38%), when compared to surgery (16%). Initial length of hospital stay (LOS) was increased after surgery, but this difference vanished over time due to stent occlusion and gastric outlet obstruction (GOO) in the interventional group resulting in repeated consultations and readmissions [10].

A major limitation of interventional biliary drainage is stent occlusion compromising long-lasting efficacy of palliation when compared to surgical procedures. A significantly higher rate of readmissions, 76.9% of which is due to recurrent jaundice and sepsis, was reported in patients treated with biliary stents ($n = 33$) when compared to patients receiving a hepaticojejunostomy and a gastrojejunostomy ($n = 23$) in a retrospective analysis in 2009 (39.4% vs. 13.0%, respectively, $p < 0.05$) [11].

The long-lasting effects of surgical palliation with low rates of recurrent jaundice and acceptable incidence of postoperative complications were further demonstrated in 118 consecutive patients with unresectable pancreatic cancer diagnosed upon surgical exploration. The most commonly performed surgical procedure was a double bypass (biliary bypass with gastrojejunostomy in 75%) with a total of 107 patients receiving a gastrojejunostomy. Overall postoperative complication rate was 37%, while wound infections were most common with 10%, cholangitis developed in 8%, and delayed gastric emptying in 8%. Hospital death occurred in 2.5%. During the follow-up, only 4% developed gastric outlet obstruction (GOO), and 2% suffered from recurrent jaundice during a mean survival after palliative surgery of 7.7 months [12]. Additionally, the beneficial long-term effects of surgical treatment of obstructive jaundice (recurrent jaundice: 8%) with acceptable perioperative morbidity (21% of early complications) have also been confirmed in other studies, and surgical palliation for unresectable pancreatic cancer by a surgical double bypass was advocated to be the first-line treatment with high success rates (>95%) in patients eligible for surgery [6, 13–15].

Furthermore, a recent meta-analysis on five randomized controlled trials comprising 379 patients comparing biliary stent placement with surgical bypass confirmed these single study data. There was no significant difference in the success rate between the different strategies ($p = 0.67$). Importantly, major complications and mortality were not increased after surgical palliation ($p = 0.14$), but surgical treatment was associated with decreased recurrence of jaundice when compared to biliary stent placement (RR 0.14, $p < 0.01$). Thus, if operative risk is moderate or low, surgical palliation should always be considered as the first choice [16].

A critical point for surgical palliation is the selection of patients being eligible for surgery. In this context, the association between the Portsmouth Physiological and Operative Severity Score for the enUmeration of Mortality and Morbidity (P-POSSUM) and cardiopulmonary exercise testing (CPET) and the incidence of

postoperative complications was recently analyzed. 50 patients were included undergoing double bypass surgery for unresectable pancreatic cancer and collected demographic data, preoperative anesthetic performance and postoperative outcome were evaluated. The P-POSSUM score was significantly increased in patients experiencing postoperative complications (median P-POSSUM physiology score: 18.7 vs. 16.5, $p = 0.005$), while the aerobic threshold was significantly lower in patients with postoperative complications (11.3 ml/kg/min vs. 14.1 ml/kg/min, $p = 0.016$). Additionally, multivariate analysis showed postoperative complications being an independent risk factor for decreased survival (OR 3.261, 0.95 CI 1.492–7.129, $p = 0.003$). Therefore, a critical patient selection is always mandatory in patients planned for surgical palliation [17].

Interestingly, if patients undergo preoperative endoscopic intervention, they have higher postoperative morbidity and mortality following palliative bypass surgery. In 204 patients receiving double bypass (77.45%), biliary bypass (18.13%) or gastric bypass (4.32%), wound infection rate (40% vs. 8.37%, $p < 0.001$), bile leak rate (28% vs. 1.76%, $p < 0.001$), and hospital mortality (8% vs. 0%, $p < 0.001$) were significantly increased after a previous biliary endoscopic intervention. Therefore, surgery should always be considered as a first step to palliate jaundice in fit pancreatic cancer patients [18].

Significant improvements in biliary decompression were achieved by the development and usage of metal stents delivering higher patency rates than plastic stents. The effect of metal stent placement ($n = 29$) for palliation in patients with unresectable pancreatic cancer in comparison with surgical palliation by either biliodigestive bypass operation ($n = 11$) or palliative Whipple's procedure ($n = 12$, defined as R1 resection with initially curative intent) was recently investigated. Indications for stenting were metastatic disease, progression during neoadjuvant treatment, age over 80 years, or if patients were unfit for resection or they refused surgery. Primary endpoints were mortality within 30 days and survival, while second endpoints were complications and biliary and intestinal patency rates. There was no mortality within 30 days reported. Median survival was 280 days (95% CI, 103–456 days), 157 days (95% CI, 0–411 days), and 647 days (95% CI, 300–993 days) in patients with stenting, bypass surgery, and Whipple's procedure, respectively ($p = 0.111$). Patency of the biliary and intestinal track was not significantly different within the groups ($p = 0.112$) [19]. These data delineated the role of interventional palliation in selected patients, especially in light of newly developed metal stents, which have also been shown to lead to improved outcomes in quality of life ($p = 0.042$) with reduced costs ($p = 0.0013$) [20].

The most common surgical approach in jaundiced patients is the use of a hepaticojejunostomy for efficient biliary drainage. However, alternatives such as a hepaticocholecystoduodenostomy are possible. In a controlled randomized trial comparing surgical drainage via hepaticocholecystoduodenostomy ($n = 10$) with drainage by Roux-en-Y choledochojejunostomy ($n = 10$), technical success was similar in both groups, but patients receiving a hepaticocholecystoduodenostomy had a shorter operative time ($p < 0.0001$), less blood loss ($p = 0.0001$), and shorter

hospital stay ($p < 0.0001$). With similar rates of recurrent jaundice, gastroduodenal obstruction, and hemorrhage, hepaticocholecystoduodenostomy might be an alternative technique for palliative biliary drainage [21].

Indications for surgical palliation also seem to change. In a large single institutional survey on 1913 patients of whom 583 underwent palliative treatment, indication for palliative bypass surgery changed from locally advanced disease vs. liver or peritoneal metastasis between 2002 and 2007 with 49.2% vs. 50.8%, respectively, to 17.2% vs. 82.7% between 2008 and 2010 ($p = 0.005$). These numbers might reflect more aggressive resection polices also in locally advanced disease and the use of potent neoadjuvant therapy regimes which convert many patients with locally unresectable disease into resectable cases [22].

Different Techniques of Interventional Drainage: Which Stent to Choose?

Endoscopic treatment of malignant biliary obstruction, especially with increasing use of self-expandable metal stents (SEMS), displays a promising approach for patients suffering from unresectable metastatic pancreatic cancer, particularly when expected survival is short, or patients are not eligible for surgery.

A randomized trial showed median patency being higher in SEMS (covered) than in plastic stents (3.6 months vs. 1.8 months, $p = 0.002$) [23]. Additionally, partially covered metal stents (pcSEMS) also have longer patency rates when compared to plastic stents (385.3 $\pm$ 52.5 days vs. 153.3 $\pm$ 19.8 days, $p = 0.006$). Interestingly, cholangitis occurred significantly more often in the plastic stent group when compared to the metal stent group (24.5% vs. 4.9%, $p = 0.029$) [24].

When comparing partially covered SEMS ($n = 51$) and uncovered SEMS ($n = 52$), patency, survival, stent dysfunction, and adverse events were not significantly different [25]. Although, stent material seems to be of relevance, since in 200 randomized patients with malignant biliary obstruction increased patency rates after 300 days were observed in covered nitinol SEMS (89%) vs. steel SEMS (77%, $p = 0.01$) [26]. Thus, SEMS seems to be superior to plastic stents, but covered stents seem to have no benefit over uncovered stents.

Readmission due to stent occlusion and associated complications (e.g., cholangitis) plays an important role for interventional drainage and thereby contributes a significant cost factor for the public health system. In a cost-efficacy analysis within a randomized trial of 219 patients receiving plastic stents ($n = 73$), partially covered ($n = 71$) and uncovered SEMS ($n = 75$) due to malignant biliary obstruction, functional patency time was significantly longer in SEMS (uncovered, 288 days, vs. partially covered, 299 days, vs. plastic, 172 days, $p < 0.005$). After 1 year of follow-up, 83% of the patients were dead (182/219), while 14% were still alive and 3% were lost during follow-up. The overall median survival in this patient cohort was 109 days, and the type of stent did not significantly change survival ($p = 0.241$). Concerning treatment cost, although initial costs were higher after the placement of a metal stent ($p = 0.001$), costs did not differ after 1 year of follow-up

($p = 0.61$). Even in patients with short survival (less than 3 months), costs were not significantly different in respect of the placed stent type [27]. Therefore, in a definitive palliative situation, a metal stent should be used and plastic stents avoided.

If stents cannot be placed endoscopically via the papilla of Vater route, alternative routes of nonanatomical biliary drainage are endoscopic ultrasound (EUS)-guided biliary drainage by either hepaticogastrostomy (HPG) or choledochoduodenostomy (CD), both using a partially covered SEMS. In a group of 49 patients with unresectable malignant biliary obstruction, 25 patients were randomized to receive HPG after failed endoscopic retrograde cholangiopancreatography (ERCP), while 24 received CD. Both HPG and CD were associated with a high technical (96% vs. 91%, $p = 0.609$) and clinical (resolution of jaundice) (91% vs. 77%, $p = 0.234$) success rate, respectively. Quality of life improved significantly in both groups, without differences in survival ($p = 0.603$). Therefore, after failed ERCP drainage, nonanatomical biliary drainage might be an option in selected patients if experience with this procedure is present [28].

Prophylactic Gastrojejunostomy for Unresectable Periampullary Cancer

Besides obstruction of the biliary track, patients with unresectable malignancy of the pancreas and especially of the pancreatic head are prone to develop gastric outlet obstruction (GOO) within the course of their disease. Even among those patients considered to have a low risk of developing GOO, the incidence lies between 10 and 15% [29, 30]. To overcome this, performance of a prophylactic gastrojejunostomy at exploration is possible and even recommended, even in asymptomatic patients, if expected survival is not very limited [31]. Whether this procedure is feasible and accompanied with acceptable morbidity and mortality was investigated in two randomized trails.

In a prospective randomized trial ($n = 87$ patients) on the efficacy of a prophylactic retrocolic gastrojejunostomy in patients who were initially planned for curative pancreaticoduodenectomy (PD) and were found to have unresectable disease at exploration, 44 patients were randomized for prophylactic gastric bypass surgery, while 43 did not receive a bypass. No difference was reported on postoperative morbidity and mortality (32% vs. 33%) nor on hospital stay (8.5 days $\pm$ 0.5 day vs. 8.0 days $\pm$ 0.5 day) or long-term survival (8.3 months for both groups) in both groups. Most importantly, 19% of the patients without gastrojejunostomy developed gastric outlet obstruction and needed further interventions, after a mean period of 2 months postoperatively. On the other hand, none of the patients with bypass surgery developed a gastric outlet obstruction ($p < 0.01$). These data strongly support the recommendation that patients with unresectable pancreatic malignancy at exploration should receive a gastrojejunostomy on a routine basis [32]. These data were reconfirmed by a randomized study comparing 65 patients with unresectable periampullary tumors in whom a double bypass (hepaticojejunostomy and retrocolic gastrojejunostomy, $n = 36$) was compared with a single bypass alone

(hepaticojejunostomy, $n = 29$). The incidence of gastric outlet obstruction during follow-up was significantly higher in patients with a single bypass when compared with patients receiving a double bypass (41.4% vs. 5.5%, $p = 0.001$). Of the 12 patients experiencing GOO after single bypass, 50% underwent a relaparotomy with a secondary gastrojejunostomy after a median time of 3.5 months after initial exploration. On the other hand, postoperative morbidity (31% vs. 28%, $p = 0.12$), length of hospital stay (11 days vs. 9 days, $p = 0.06$), and median survival (7.2 months vs. 8.4 months, $p = 0.15$) were not significantly different between the double bypass group and the single bypass group, respectively. Quality of life was also not different between the procedures [33].

The importance of a prophylactic gastrojejunostomy in palliative pancreatic cancer was further underlined by Gurusamy et al. who pooled the abovementioned studies in a meta-analysis and confirmed a significant reduction of gastric outlet obstruction (2.5% vs. 27.8%, RR 0.10, 95% CI 0.03–0.37) in patients undergoing a prophylactic gastrojejunostomy compared to patients without gastric bypass. On the other hand, survival (HR 1.02, 95% CI 0.84–1.25), as wells as morbidity, and quality of life were unchanged. Naturally, interventions involving a gastrojejunostomy had an increased operating time (MD 45 min, 95% CI 21.39–68.61) [34].

Furthermore, underlining the efficacy of surgical palliation and delineating the timing of surgical palliation, van Wagensveld and coworkers investigated the effects of palliative surgical procedures in 126 patients suffering from unresectable pancreatic cancer receiving a Roux-en-Y hepaticojejunostomy and a gastrojejunostomy. Indication for palliative approach was irresectability at exploration in 44 patients, failure of endoscopic treatment in 43, GOO in 28 patients, and other reasons in 11 patients. One hundred-eighteen patients received a double bypass, six a single biliary bypass, and two only a gastrojejunostomy. Most patients in this study received a prophylactic gastrojejunostomy (77%), while only a minority of patients (23%) was symptomatic at the time of surgery. Complications developed in 10% of the patients, and the 30-day mortality was 1%. Delayed gastric emptying (DGE) developed in 14% of the patients. Patients that were symptomatic for GOO at the time of surgery had an increased risk of developing DGE when compared to the asymptomatic patients prior to surgery (25% vs. 12%, $p < 0.05$). Late obstruction occurred in 11% at a median time of 141 days (21 – 356 days) after treatment. This study further underlines surgical palliation being an effective method associated with low morbidity and mortality. Most notably, gastric bypass surgery should also be performed in asymptomatic patients if irresectability is found during exploration [35].

The surgical principle to perform a gastric bypass in palliation of GOO is of pivotal importance. A Roux-en-Y gastrojejunostomy (GE) by antecolic latero-lateral gastrojejunostomy after dissection of the jejunum 20 cm after the ligament of Treitz without transection of the stomach ($n = 21$) is superior compared with a conventional GE (hand-sewn side-to-side antecolic gastroenterostomy, 20 cm after the ligament of Treitz) ($n = 20$) in non-jaundiced patients with unresectable pancreatic cancer. The time to nasogastric tube removal ($p < 0.001$), time to liquid ($p < 0.001$), soft ($p < 0.001$), and regular diet ($p < 0.002$), as well as need for prokinetics ($p = 0.025$) were significantly reduced. Additionally, hospital stay was

significantly reduced (7.7 days vs. 9.6 days, $p = 0.006$) after Roux-en-Y gastroenterostomy [36].

Most of these gastric bypass procedures can nowadays be performed laparoscopically. Laparoscopic gastrojejunostomy for palliation of malignant gastric outlet obstruction gives no advantage in regard to operation time ($p = 0.75$), but intraoperative blood loss ($p = 0.0001$), time to oral food intake ($p = 0.04$), and incidence of delayed gastric emptying ($p = 0.04$) were significantly reduced when compared to open surgery [37].

Interventional Approaches for Gastric Outlet Obstruction

Gastric outlet obstruction can also be treated by endoscopic stent placement. Comparing gastrojejunostomy ($n = 18$) with endoscopic duodenal stent placement ($n = 21$) in a randomized controlled trial (SUSTENT study), food intake, according to a standardized GOO scoring system (GOOSS), was improved more rapidly after stenting than after surgery (GOOSS $\geq$ 2: 5 days vs. 8 days, $p < 0.01$), while long-term effects were significantly better after gastrojejunostomy compared with stenting (GOOSS $\geq$ 2 for 72 days vs. 50 days, $p = 0.05$). Additionally, major complications ($p = 0.02$), recurrent obstruction ($p = 0.02$), and re-interventions ($p < 0.01$) occurred more frequently after stenting compared to surgery. Interestingly, post-interventional complications were similar when eliminating stent occlusion ($p = 0.4$). Furthermore, median survival ($p = 0.19$) and QOL were equal between the groups [38].

Cost analysis in this study revealed that initial costs were significantly higher in the group receiving a gastrojejunostomy ($p < 0.001$), an effect mainly due to longer hospital stay after surgery (15 days vs. 7 days, $p = 0.04$). However, the follow-up costs were equal between the two groups ($p = 0.7$). Overall, total costs per patient were higher in the surgical group, when compared to the interventional group ($p = 0.049$). However, cost-effectiveness ratio showed only increased cost of 164€ per extra day without GOO (GOOSS $\geq$ 2). Based on these studies, gastrojejunostomy is the treatment of choice if expected survival is not very compromised (>2 months), although costs might be higher. Therefore, a gastrojejunostomy should be preferred in light of improved long-term outcome in patients with gastric outlet obstruction [39].

Pain Management in Advanced Pancreatic Cancer

Many patients with advanced pancreatic cancer suffer from pain located in the upper abdomen and the back resulting from neural invasion and neurogenic inflammation, which drastically compromises quality of life [40]. Thus, adequate pain treatment is mandatory in most pancreatic cancer patients. In general, first-line treatment is performed by oral analgesics, ideally in accordance to the WHO guidelines. Further steps in severe pain treatment are neurolytic interventions leading to pain reduction

by denervation. This can be performed at different levels (e.g., at the thoracic level with splanchnicectomy or at the abdominal level by neurolysis of the celiac plexus) leading to a reliable pain control. On the other hand, those interventions are capable of giving rise to associated complications due to denervation like diarrhea, orthostatic hypotension, or most seriously paraplegia. The advantages, disadvantages, and optimal timing of interventions are discussed in the following sections [41, 42].

One method of pain relief is neurolysis of the celiac plexus, which can be performed in a uni- or bilateral manner. In a randomized controlled trial, no differences between unilateral and bilateral celiac plexus neurolysis (50% ethanol + 0.25% bupivacaine) with percutaneous anterior abdominal ultrasound guidance in unresectable GI cancer were observed. The onset of pain relief was not dependent of uni- or bilateral injection technique ($p = 0.17$) as was not patients' satisfaction after treatment (64.67 ± 26.06 vs. 67.00 ± 26.51, scale: 0–100, $p = 0.73$). Additionally, post-interventional complications such as diarrhea (40% vs. 33.3%, $p = 0.59$) or hypotension (13.3% vs. 10%, $p = 1.00$) were similarly frequent comparing uni- and bilateral injection technique. Furthermore, long-term results revealed comparable outcomes with pain scores (numerical rating scale of 0–100) of 18.7 ± 12.8 for the unilateral and 20.0 ± 11.17 for the bilateral technique ($p = 0.53$) [43].

Besides transcutaneous sonography guidance, neurolysis of the celiac plexus can be achieved using computed tomography (CT) or EUS guidance. In a randomized trial, celiac plexus blockade by EUS guidance ($n = 10$), performed by injection of 10 ml of bupivacaine (0.75%) and 3 ml of triamcinolone (40 mg), achieved pain reduction in 50% of the patients, while by CT guidance ($n = 8$) only 25% of the patients experienced sufficient pain relief. Additionally, cost analysis revealed that the EUS technique was less costly when compared to the CT technique [44].

Beside the route of injection, also the target of injection plays an important role. Celiac neurolysis can either be directed selectively against the celiac plexus or the celiac ganglia. When in a randomized controlled trial, EUS-guided celiac plexus neurolysis ($n = 34$) was compared with celiac ganglia neurolysis ($n = 34$); patients receiving blockade of the celiac ganglia had a significantly higher response rate 7 days after the intervention when compared to patients receiving block of the celiac plexus (73.5% vs. 45.5%, $p = 0.026$). Furthermore, the complete response rate (pain level of 1 or lower on a scale of 0–10) was significantly higher after blockade of the celiac ganglia, compared to neurolysis of the celiac plexus (50.0% vs. 18.2%, $p = 0.01$). Adverse events or duration of pain relief was similar in both groups indicating that celiac ganglia blockade is superior to celiac plexus blockade for palliation of pain in advanced pancreatic cancer [45].

In addition to celiac plexus blockade, palliation of pain can also be achieved by splanchnicectomy, either performed bilaterally or unilaterally by thoracoscopy. When prospecting the effect of bilateral thoracoscopic splanchnicectomy by transection of the nerve in patients suffering from pancreatic cancer ($n = 23$) or chronic pancreatitis ($n = 21$) in the follow-up (3 months for cancer, 43 months for chronic pancreatitis), a long-lasting pain relief of $\geq$50% (visual analog scale (VAS) scale, 0–10) was demonstrated, already beginning in the first postoperative week. This

was associated with decreased analgesic medication consumption, while exocrine (secretin test) and endocrine pancreatic functions (basal serum glucose, plasma insulin, C-peptide) were unaffected. There was no procedure-related death, but nine patients required a thoracotomy because of bleeding [46]. A significant pain relief can also be achieved by left-sided thoracoscopic splanchnicectomy via nerve transection. The sufficient effect of pain relief by thoracoscopic splanchnicectomy was verified in 26 patients with advanced pancreatic cancer, where a significant reduction of pain was achieved in all patients. After 1 week, pain scores were significantly reduced (8.54 vs. 1.77, scale: 0–10, $p < 0.001$) when compared to scores prior to the operation. Additionally, interference with general activity decreased significantly after the intervention (8.42 vs. 2.38, $p < 0.0001$). Simultaneously, the analgesic treatment regime improved, and patients did not depend on opioid consumption [47].

Thus, thoracoscopic splanchnicectomy is a beneficial intervention for amelioration of pain without compromising exocrine and endocrine pancreatic function.

When the effect of intraoperative splanchnicectomy using 50% alcohol ($n = 65$) vs. saline 0.9% ($n = 72$) was studied (double-blinded study) in unresectable pancreatic cancer patients, the postoperative complication rate (35% vs. 34%) and length of hospital stay (13.8 days vs. 13.9 days) were not significantly different. Importantly, a significant reduction of pain was observed in the alcohol group at 2, 4, and 6 months of follow-up ($p < 0.05$). Interestingly, alcohol injection significantly reduced the pain pattern in both patients that had pain before the intervention and patients without pain before the intervention ($p < 0.05$). Furthermore, patients with pre-interventional pain experienced a prolonged survival after alcohol injection when compared to saline injection ($p < 0.0001$). During long-term follow-up, 10% of the patients in the alcohol group needed further intervention by percutaneous celiac axis block compared to 12% in the saline group. Most notably, the time to re-intervention was significantly longer after alcohol treatment when compared to saline (11.8 ± 3.2 months vs. 4.0 ± 1.1 months, $p < 0.05$) [48].

An additional analysis of those patients in a follow-up study verified the positive impact on survival (9.15 months vs. 6.75 months, $p < 0.05$) after alcohol treatment. Additionally, the patients are divided into two groups according to their mood state (scale 0–10), those with highly negative mood suffered more pain (VAS 0–10: 4.33 vs. 2.52, $p < 0.0001$) and experienced more interference of daily activities (scale 0–10: 4.94 vs. 3.07, $p < 0.0001$) when compared to patients with lower negative mood, further underlining the beneficial effects of pain relief [49].

When both available neurolytic approaches, celiac plexus ($n = 19$) and splanchnic nerve blockade ($n = 20$) were compared in a randomized trial in patients suffering from carcinoma of the body or the tail of the pancreas a significantly higher reduction of pain after splanchnic nerve blockade was observed 14 weeks after the intervention ($p < 0.001$). Additionally, in the 4-week follow-up, patients' satisfaction was significantly higher after splanchnic nerve blockade ($p = 0.003$). Alongside with this, patients with splanchnic nerve blockade had a significantly increased reduction in opioid consumption and a longer survival than patients receiving celiac plexus blockade (68.85 ± 7.3 days vs. 45.37 ± 5.82 days, $p = 0.0072$) [50].

Another important aspect in interventional pain treatment is the correct timing and interplay with pharmacological analgesic treatment. In this context, the effect of timing of celiac plexus blockade on pain relief in patients with pancreatic carcinoma receiving pharmacological pain treatment before blockade of the celiac plexus compared to patients receiving pharmacological treatment after blockade of the plexus was investigated. At all time points, pain scores (VAS) were significantly lower after treatment by neurolytic celiac plexus blockade ($p < 0.0001$), supporting the efficacy of this approach. However, pain scores were significantly lower in patients who received pharmacological pain treatment before plexus blockade ($p < 0.0001$) alongside with increased quality of life (QLQ-C30) ($p < 0.0001$). Therefore, a pharmacological treatment of pain prior to plexus blockade seems to be more effective than vice versa [51]. In addition, early celiac plexus blockade by EUS is associated with an increased pain relief ($p = 0.01$) and a tendency toward a reduction of morphine consumption ($p = 0.10$). Therefore, early plexus neurolysis should be considered in patients with unresectable pancreatic cancer during diagnostic or staging EUS [52].

However, limitation of interventional splanchnicectomy or celiac plexus blockade is also reported [53]. Comparison of opioid analgesics, celiac plexus blockade, and thoracoscopic splanchnicectomy for pain relief in unresectable malignancies of the pancreas revealed no difference in pain relief after 2 months of follow-up, raising the question of the value of invasive pain interventions, in light of potential complications [53]. Additionally, comparison of patients receiving celiac plexus blockade (0.5% bupivacaine/100% alcohol) with patients treated with systemic analgesics and sham injection revealed no effect on opioid consumption ($p = 0.93$) and QOL ($p = 0.46$) besides, however, a significant ($p = 0.005$) and long-lasting ($p = 0.01$) amelioration of pain [54].

Palliative Pancreaticoduodenectomy

As imaging modalities proceed and reporting of preoperative CT imaging is more standardized, preoperative staging of pancreatic cancer is becoming more accurate, and resectability can in most cases be determined very clearly [55]. On the other hand, resection policies in locally advanced pancreatic cancer are getting more aggressive, especially in light of higher resection rates and survival after potent neoadjuvant treatment protocols like FOLFIRINOX [56]. Nevertheless, in some cases, a surgeon can end in a situation where complete resection of the malignancy is not feasible, and surgery ends in an R2 resection. This is an unfavorable situation because R2 resections are associated with a longer operative time (397.5 min vs. 240 min, $p < 0.0001$), higher blood loss (750 ml vs. 200 ml, $p < 0.0001$), higher morbidity (47.4% vs. 21.7%, $p = 0.0197$), more relaparotomies (13.2% vs. 0%, $p = 0.0163$), and longer hospital stay (12.5 days vs. 10.5 days, $p = 0.011$) compared to patients undergoing palliative bypass surgery. Postoperative mortality (7.9% vs. 2.2%, $p = 0.3239$) is not significantly different. However, there is also no improvement in median survival after R2 resection compared with bypass surgery

(10.7 months, $p = 0.656$) [57]. The missing impact on survival after R2 resection with increased morbidity (RR of 1.75 ($p < 0.0001$)) and mortality (RR 2.98 ($p = 0.009$)) was further validated in a meta-analysis comprising 399 patients, of which 138 received an R2 resection and 261 bypass surgery [58]. Thus, tumor mass reduction (R2 resection) is not an intended treatment option for pancreatic cancer.

On the other hand, patients receiving an unintended R2 resection have no major disadvantage, and therefore aggressive exploration of pancreatic cancer should be performed whenever possible.

In contrast to R2 resections, nonradical resections (R1 with tumor 1 mm from the resection margin) offer a survival benefit compared to palliative bypass surgery. Bypass surgery was associated with lower morbidity and hospital stay. However, median survival was significantly longer after R1 resection (17.4 months), when compared to R2 (8.5 months) and bypass surgery (9 months, $p < 0.001$), and survival rates within 1 year were significantly improved after R1 resection (71% for R1, 46% for R2, and 32% for bypass surgery; $p < 0.001$). These findings were also confirmed in a systematic review including eight studies with 1,535 patients. After R1 or R2 resection, morbidity was increased (both 48%) when compared to bypass procedures (30–34%). However, median survival was significantly longer in R1-resected patients, and therefore exploration with R0/R1 resection should always be aspired to, when compared to bypass surgery [59].

Conclusion

In conclusion, surgical palliation for obstructive jaundice in unresectable pancreatic cancer is a potential, feasible, and safe treatment option. Recurrent jaundice is significantly reduced after surgery when compared to interventional approaches (endoscopic stents, percutaneous transhepatic stents). In patients with poor expected survival of self-expandable metal stents display a promising alternative for palliation of obstructive jaundice.

When interventional biliary drainage is inevitable, self-expandable metal stents show higher patency compared to plastic stents and are cost effective even in patients with short survival. Additionally, self-expandable metal stents were also associated with reduced rates of cholangitis when compared to plastic prosthesis.

If irresectability is evident during exploration, performance of a prophylactic gastrojejunostomy is recommended to overcome the potential risk of GOO even in asymptomatic patients. There is evidence that a Roux-en-Y gastrojejunostomy is superior to a conventional gastroenterostomy in light of faster postoperative recovery. A recent RCT showed surgical palliation being superior to interventional palliation by duodenal stenting and being the treatment of choice if survival is longer than 2 months. If patients are eligible, laparoscopic palliation might be considered because of faster postoperative recovery.

Neurolytic procedures, either by splanchnicectomy or by neurolysis of the celiac plexus or ganglia, provide a potential treatment option for pain associated with advanced malignancy of the pancreas. Splanchnicectomy seems to be superior to

interventions affecting the celiac plexus. However, sufficient pharmacological pain relief should be achieved prior to neurolytic interventions. Blockade of the celiac plexus by EUS can be considered also in the early time points of irresectability. Additionally, celiac ganglia blockade is superior to celiac plexus blockade.

In light of potent neoadjuvant treatment, patients with likelihood of non-curative resection should undergo pretreatment. Following neoadjuvant therapy, exploration and evaluation of resectability should be intended whenever possible. Planned R2 resections should be avoided due to increased postoperative complications without survival benefit, unless a point of no return (e.g., dissection of the pancreatic neck) has been passed.

Cross-References

- ▶ Arterial Resection in Pancreatic Cancer
- ▶ Borderline Resectable Pancreatic Cancer
- ▶ Chemotherapy for Advanced Pancreatic Cancer
- ▶ Interventional Radiology for Pancreatic Cancer
- ▶ Palliative Management of Pancreatic Cancer
- ▶ Therapeutic Endoscopy in the Management of Pancreatic Cancer
- ▶ Treatment of Recurrent Pancreatic Cancer After Surgery

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Chemotherapy for Advanced Pancreatic Cancer

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Contents

Conflicts of Interest: Dr. Sclafani, Dr. Okines and Dr. Ratnayake have no potential conflict of interest to declare. Dr. Chau received honoraria from Roche, Merck Serono, Sanofi-Aventis, Pfizer, Eli-Lilly and Taiho and had advisory roles with Roche, Merck Serono, Sanofi-Aventis, Bristol Myers Squibb, Eli-Lilly, Novartis and Gilead Science. He also received research funding from Merck-Serono, Novartis, Roche and Sanofi Aventis. Professor Cunningham has previously received honoraria for presentations and advisory boards from Roche, Merck Serono, Amgen and Sanofi-Aventis and has received research funding from Roche, Merck Serono, Amgen, Sanofi-Aventis, Celgene, Novartis, AstraZeneca, MedImmune, Bayer, and Merrimack.

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Abstract

Gemcitabine has been key to the management of advanced pancreatic cancer since its superiority over 5-fluorouracil (5-FU) for clinical benefit, and overall survival (OS) was established in a clinical trial published in 1997. The addition of the tyrosine kinase inhibitor (TKI), erlotinib, to gemcitabine has shown a modest but statistically significant improvement in OS compared to gemcitabine alone, making it a new standard for advanced pancreatic cancer. However, limited access to targeted agents due to high costs has meant that erlotinib is not available to all patients. A meta-analysis has demonstrated that the combination of the oral fluoropyrimidine, capecitabine, and gemcitabine (GemCap) has an OS benefit of a similar magnitude to combination with erlotinib; therefore, it is a very good alternative for patients without access to funding for the higher-cost drug and is an accepted standard at many centers. Pooled analyses of trials combining gemcitabine with platinum agents have similarly demonstrated an advantage over single-agent gemcitabine offering a further therapeutic option. Recently, the therapeutic armamentarium for advanced pancreatic cancer has been enriched by two additional chemotherapy regimens including a combination of 5-FU, folinic acid, irinotecan, and oxaliplatin (FOLFIRINOX) and a combination of gemcitabine plus nab-paclitaxel. Both regimens have been demonstrated to be superior to gemcitabine alone in terms of response rate, progression-free survival, and OS and have become standard first-line treatments for patients with good performance status. Also, evidence has increasingly emerged suggesting that chemorefractory patients may benefit from the use of second-line chemotherapy. Clinical trials have shown that combining 5-FU and folinic acid with either oxaliplatin or nanoliposomal irinotecan can improve OS following progression to first-line gemcitabine-based therapies. Nevertheless, despite recent advances in medical oncology, survival from advanced pancreatic cancer remains poor and significant breakthroughs are urgently needed.

Keywords

Advanced pancreatic cancer · Metastatic pancreatic cancer · Chemotherapy · Targeted therapy · Chemoradiotherapy

Introduction

Although only the tenth most commonly diagnosed cancer, pancreatic cancer was the fourth most common cause of cancer death in Europe in 2012 [1], suggesting that its treatment is lagging far behind that of more common cancers. Presentation is typically late with either inoperable locally advanced or metastatic disease.

This, combined with the aggressive and relatively chemotherapy- and radiotherapy-resistant underlying tumor biology, makes pancreatic cancer a particular oncological challenge. Survival for patients with advanced disease is poor at a median of 2.5–3.5 months with supportive care alone. Of the patients who undergo curative surgery, the majority will eventually relapse, with 5-year survival ranging from 10.4% to 28.8% in resected patients, with or without adjuvant chemotherapy, respectively [2, 3]. Palliative chemotherapy improves survival compared to supportive care alone [4], with newer combination regimens showing more activity than gemcitabine monotherapy, although the benefits in duration of survival are modest. In contrast, the role of chemoradiation either as upfront or consolidation treatment after systemic chemotherapy in locally advanced disease is not clear. This chapter will discuss the current therapeutic options for patients with advanced pancreatic cancer and review data from clinical trials of chemotherapeutic agents and targeted therapies in this setting.

First-Line Chemotherapy

5-FU Monotherapy

Continuous 5-FU infusion demonstrated activity with moderate toxicity in a small phase II study of 16 patients with advanced pancreatic cancer in 1988. The response rate was reported as 19%, with a further 50% of patients achieving stable disease [5]. A 1991 phase II trial demonstrated that bolus 5-FU with leucovorin was also active in advanced pancreatic cancer, reporting three partial responses in the 42-patient study (7%) and a 6.2-month median OS in patients treated on a weekly schedule for 6 weeks out of an 8-week cycle [6]. In contrast, a concurrently reported phase II trial of high-dose infused leucovorin and bolus 5-FU demonstrated significant toxicity, mainly stomatitis and diarrhea, coupled with no partial or complete responses and a median survival of only 10 weeks [7], showing the fine balance between therapeutic dosing and toxicity that is required to achieve survival benefits. Protracted venous infusion (PVI) 5-FU was compared to a combination of PVI 5-FU and mitomycin C in a randomized phase III trial of 209 patients, but despite an improvement in response rate in the combination arm (17.6% vs. 8.4%, $p = 0.04$), the improvements in median failure-free survival (3.8 vs. 2.8 months) and overall survival (6.5 vs. 5.1 months) failed to reach statistical significance [8]. Although 5-FU is clearly an active agent in this setting, neither 5-FU monotherapy nor combination with mitomycin C would be considered a standard treatment regimen in advanced pancreatic cancer.

Gemcitabine Monotherapy

There was no internationally accepted standard regimen for advanced pancreatic cancer until a randomized trial demonstrated improved clinical benefit and survival

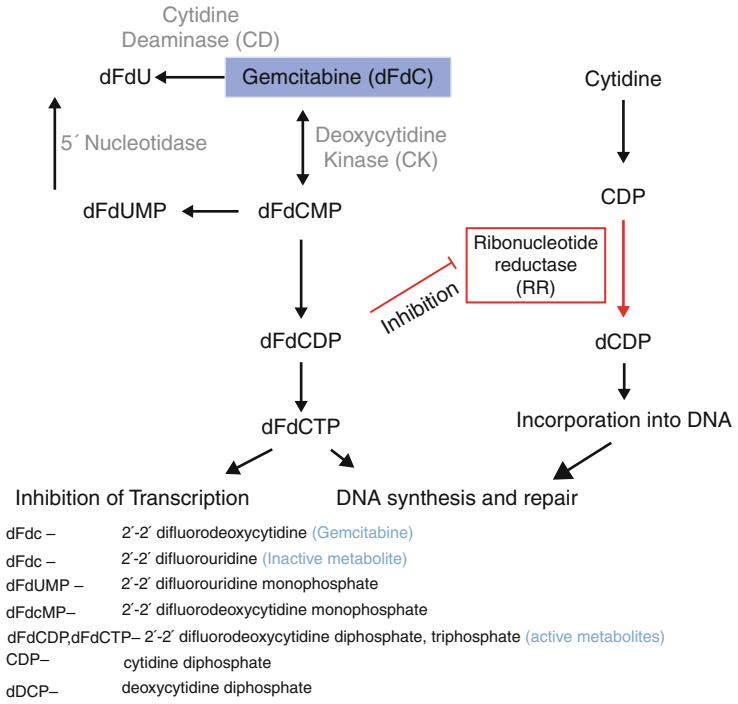

Fig. 1 Gemcitabine metabolism and action

from gemcitabine monotherapy compared to 5-FU in the first-line setting [9]. Gemcitabine hydrochloride is metabolized intracellularly to nucleoside analogues (Fig. 1) which inhibit DNA synthesis and induce apoptosis (programmed cell death). One hundred twenty-six patients with symptomatic advanced pancreatic cancer were randomized to receive a weekly 30-min infusion of 5-FU, or gemcitabine for 7 weeks followed by 1 week off, then weekly for 3 weeks out of a 4-week cycle. A clinical benefit was defined as an improvement in at least one of the following: pain and analgesic requirements, Karnofsky performance status (PS), or weight, sustained for at least 4 weeks without deterioration in any of the other factors. Twenty-three point eight percent of patients treated with gemcitabine achieved a clinical benefit compared to only 4.8% of those treated with 5-FU ($p = 0.0022$). OS was also statistically significantly better in the gemcitabine arm, although the actual benefit was relatively small (median 5.65 months vs. 4.41 months, $p = 0.0025$).

Common gemcitabine toxicities include myelosuppression, nausea and vomiting, peripheral edema, fatigue, fever, and flu-like symptoms. Gemcitabine is usually administered as a weekly infusion over 30 min for 3 weeks out of a 28-day cycle at a dose of 1,000 mg/m^2, often after 7 weekly doses in an initial 8-week cycle.

Gemcitabine monotherapy remains the standard of care for frail patients (i.e., ECOG PS 2) in many centers, with response rates of 5.6–17.3%, disease stabilization rates around 40% and median OS of 4.6–7.2 months in phase III trials using gemcitabine monotherapy as the control arm [10–15]. Of note, these response and survival rates have been achieved in selected clinical trial populations and therefore cannot be extrapolated to patients of poor performance status, or those with severe renal or hepatic dysfunction.

Fixed Dose Rate Gemcitabine

Deoxycytidine kinase, the enzyme which initiates phosphorylation of gemcitabine, and therefore eventual conversion of gemcitabine to the active gemcitabine triphosphate, has saturable kinetics. Attempts have been made to maximize conversion of gemcitabine using a fixed dose rate (FDR) delivery of 10 mg/m^2/min, rather than the standard 30-min infusion. This has been investigated in the phase I setting, where a total dose of 1,500 mg/m^2 was recommended, and in the randomized phase II setting, with no significant improvement in time to treatment failure (the primary endpoint) demonstrated when compared to a higher dose of gemcitabine given over 30 min (median 2.1 vs. 1.8 months, $p = 0.09$) with increased hematological toxicity seen in the FDR arm. However, a median OS difference was demonstrated (8.0 vs. 5.0 months, $p = 0.013$); therefore, the use of FDR gemcitabine was investigated within combination chemotherapy regimens. A single-arm phase II trial of FDR gemcitabine in combination with 5-FU demonstrated a median OS of 5.7 months; therefore, the combination was not further evaluated. In contrast, a very promising median OS of 9.2 months was seen for FDR gemcitabine (1,000 mg/m^2/100 min) in combination with oxaliplatin 100 mg/m^2 (GEMOX) in a randomized phase II study. This led to the three-arm phase III trial of 832 patients randomized to receive gemcitabine (1,000 mg/m^2/30 min), FDR gemcitabine (1,500 mg/m^2/150 min), or GEMOX (as per the phase II schedule), in which a nonsignificant trend toward superiority of FDR gemcitabine compared to gemcitabine standard administration was demonstrated (median OS 6.2 vs. 4.9 months, HR 0.83, 95% CI 0.69–1.00, $p = 0.04$) [16]. The trend toward superiority of the combination regimen GEMOX also failed to reach statistical significance compared to standard gemcitabine monotherapy (median 5.7 vs. 4.9 months, HR 0.88, 95% CI 0.73–1.05, $p = 0.22$). The median OS from the combination regimen was no better than that achieved with FDR gemcitabine alone (median survival 5.7 months vs. 6.2 months and 1-year survival 21% vs. 22%, respectively, confirming that FDR gemcitabine may be a useful treatment strategy).

Gemcitabine Combination Chemotherapy

The modest benefits provided by a single-agent approach led investigators to focus on potential combination chemotherapy, investigating whether doublet or triplet regimens could further improve survival. A similar approach had been successfully

employed in other gastrointestinal malignancies resulting in significant improvements in outcomes in the advanced disease setting.

Gemcitabine with 5-FU

The logical initial combination of chemotherapy agents to be investigated was gemcitabine with 5-FU, since both drugs had shown activity in pancreatic cancer and had individually demonstrated improvements in OS and clinical benefit. Additionally, both in vitro and in vivo studies in pancreatic cell lines have demonstrated a synergistic effect between gemcitabine and 5-FU, suggesting that the two drugs interfere with pyrimidine synthesis and catabolism at different levels (Figs. 1 and 2).

Several phase II studies have combined either bolus or infused 5-FU with gemcitabine, using a variety of regimens. Response rates vary from 5% to 31%, while OS from this combination ranges from 4 to 13 months. The combination proved to be well tolerated in these studies, with the common toxicities being neutropenia, thrombocytopenia, stomatitis, diarrhea, and hand-foot syndrome. However, neither the bolus 5-FU nor the continuously infused 5-FU combination regimens have been shown to be superior to gemcitabine alone.

The first randomized study of gemcitabine in combination with bolus 5-FU was attempted by the Eastern Cooperative Oncology Group (ECOG), where 327 patients

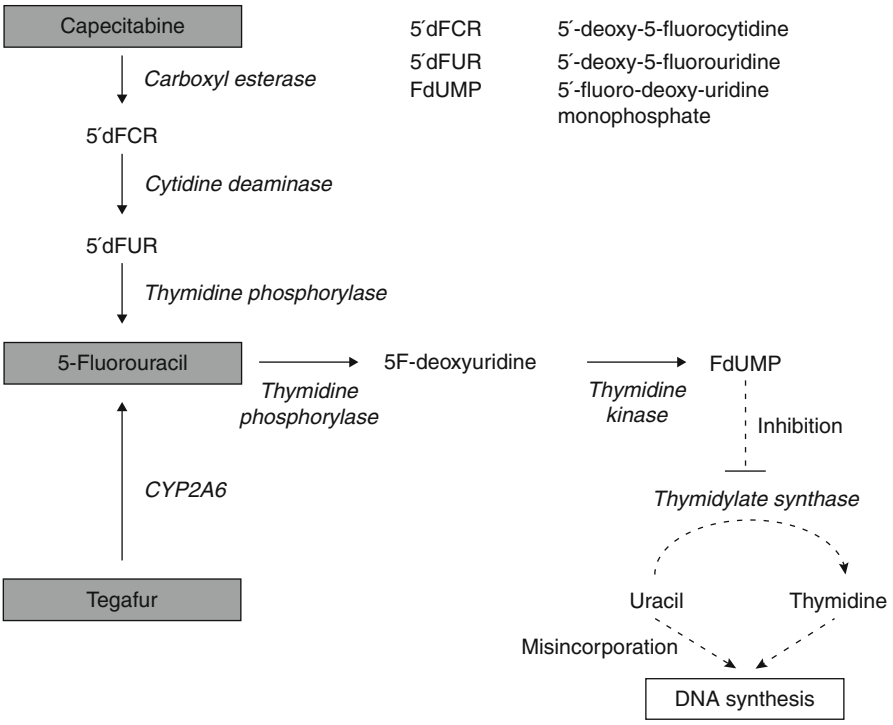

Fig. 2 5-Fluorouracil and its prodrugs

were randomly assigned to weekly gemcitabine or gemcitabine plus bolus 5-FU [11]. The median survival in the gemcitabine alone arm was 5.4 months, compared to 6.7 months for the combination group. However, statistical significance was not reached ($p = 0.09$), with the 1-year survival being identical in the two groups. A more detailed analysis of the two cohorts revealed an imbalance in the performance status of the patients and the distribution of disease. The combination arm cohort had more patients of ECOG PS 1, and patients with tumors in the pancreatic body, but fewer patients with distant metastases. When the survival analysis was adjusted to take account of these variations, statistical significance was reached, but despite a statistically significant improvement in progression-free survival (PFS) in the combination arm compared to the single-agent arm (3.4 months compared to 2.2 months, respectively, $p = 0.022$), the authors concluded that there is no clinically meaningful advantage to combining gemcitabine with 5-FU. Grade 3 and 4 toxicities were predominantly hematological or gastrointestinal in both arms, with a slightly increased rate in the combination cohort, which did not reach statistical significance (Table 1).

A second phase III study by Riess et al., which randomized 466 patients to gemcitabine with or without continuously infused 5-FU, and a randomized phase II study by the Italian Oncology Group for Clinical Research (GOIRC), using an alternative gemcitabine regimen with or without continuously infused 5-FU in 91 patients, both showed no advantage of combination chemotherapy over single-agent gemcitabine [17, 18]. In fact, both trials reported reduced median survival rates for the combination arms compared to the gemcitabine monotherapy arm (5.85 vs. 6.2 months [$p = 0.68$] and 6.9 vs. 7.2 months for the German and Italian studies, respectively). The German phase III study also failed to show any improvement in median time to progression (TTP), which was 3.5 months in both arms.

A meta-analysis of the data from these three randomized trials has confirmed that no significant advantage is afforded by the combination of gemcitabine and 5-FU over single-agent gemcitabine (HR 0.98, 95% CI 0.86–1.11). Although suggestions have been made that altering the dosing regimens may provide an improvement of outcomes, the general acceptance is that any improvement would be modest.

Gemcitabine and Capecitabine

Capecitabine (Xeloda™) is an oral fluoropyrimidine, which is selectively metabolized in tumor cells to 5-FU via a three-step enzymatic conversion process (Figs. 2 and 3). Single-agent capecitabine in chemotherapy-naïve, advanced pancreatic cancer patients has been evaluated in a phase II study, demonstrating a partial response rate of 7.3% and a clinical benefit rate of 24%, similar to single-agent 5-FU in the same setting. An initial phase I/II dose escalation study combining gemcitabine with capecitabine recruited 36 patients. The reported response rate was 18.5%, median OS 6.3 months, and 1-year survival 33%. Several randomized phase II and phase III studies have since been conducted using gemcitabine with or without capecitabine. Two phase II studies reported encouraging OS rates of 9.5 months and 9.0 months, although these were not achieved in larger phase III trials (Table 2).

Table 1 Randomized trials of gemcitabine and 5-fluorouracil (FU) in advanced pancreatic cancer

Study	Phase	Treatment	No. of patients	Median OS (months)	Median TTP (months)	Response rate (%)
Di Costanzo et al. (2005) [18]	II	Gem 1,000 mg/m^2 weekly for 7 weeks, then 1-week rest, followed by weekly for 3 weeks every 4 weeks + FU 200 mg/m^2/day CI weeks 1–6, then daily for 3 weeks every 4 weeks	43	6.9	4.2[a]	11
		Gem 1,000 mg/m^2 weekly for 7 weeks, then 1-week rest, followed by weekly for 3 weeks every 4 weeks	48	7.2	3.2	8
Berlin et al. (2002) [11]	III	Gem 1,000 mg/m^2 + FU 600 mg/m^2 d1,8,15 every 28 days	160	6.7 ($p = 0.09$)	3.4[a] ($p = 0.022$)	6.9
		Gem 1,000 mg/m^2 d1,8,15 every 28 days	162	5.4	2.2[a]	5.6
Riess et al. (2005)	III	Gem 1,000 mg/m^2 + LV 200 mg/m^2 + FU 750 mg/m^2 24 h infusion weekly for 4 weeks every 6 weeks	230	5.85 ($p = 0.68$)	3.5 ($p = 0.44$)	4.8
		Gem 1,000 mg/m^2 weekly for 7 weeks, then 1-week rest, followed by weekly for 3 weeks every 4 weeks	236	6.2	3.5	7.2

[a]Progression-free survival not TTP

Table 2 Randomized trials of gemcitabine and capecitabine in advanced pancreatic cancer

Study	Phase	Treatment	No. of patients	Median OS (months)	Median TTP (months)	Response rate (%)
Scheithauer et al. (2003)	II	Gem 2,200 mg/m^2 d1 + cape 2,500 mg/m^2/day d1-7 every 14 days	41	9.5	5.1	17
		Gem 2,200 mg/m^2 d1 every 14 days	42	8.2	4.0	14
Boeck et al. (2008)	II	Gem 1,000 mg/m^2 d1,8 + cape 825 mg/m^2 BD d1-14 every 21 days	58	9.0	5.7[a]	25
		Gem 1,000 mg/m^2 d1,8 + oxaliplatin 130 mg/m^2 d1 every 21 days	59	6.9	3.9[a]	13
		Cape 1,000 mg/m^2 BD d1-14 + oxaliplatin 130 mg/m^2 d1 every 21 days	57	8.1	4.2[a]	13
Hermann et al. (2007)	III	Gem 1,000 mg/m^2 d1,8 + cape 650 mg/m^2 BD d1-14 every 21 days	160	8.4 ($p = 0.234$)	4.3[a] ($p = 0.103$)	10.0
		Gem 1,000 mg/m^2 weekly for 7 weeks, then 1 week rest, followed by weekly for 3 weeks every 4 weeks	159	7.2	3.9[a]	7.8
Cunningham et al. (2008)	III	Gem 1,000 mg/m^2 d1,8,15 + cape 1,660 mg/m^2/day d1-21 every 28 days	267	7.1 ($p = 0.080$)	5.3[a] ($p = 0.004$)	19.1 ($p = 0.034$)
		Gem 1,000 mg/m^2 weekly for 7 weeks, then 1 week rest, followed by weekly for 3 weeks every 4 weeks	266	6.2	3.8[a]	12.4

[a]Progression-free survival

The two phase III studies evaluating the gemcitabine plus capecitabine combination (GemCap) used different dosing regimens for the capecitabine, which might explain the difference in their reported results [14, 19]. The Swiss group demonstrated a trend toward superiority of the combination, but no significant difference in the median survival between the two arms (8.4 months for the combination arm compared to 7.2 months in the gemcitabine alone arm, $p = 0.234$) [14]. Similarly, there was no statistically significant improvement in PFS with the addition of capecitabine (4.3 months for the combination arm vs. 3.9 months, $p = 0.103$). A post hoc subgroup analysis of the patients with Karnofsky PS of 90–100% ($n = 84$) showed a statistically significant improvement in OS with capecitabine in that subgroup (median OS 10.1 months for the combination arm, 7.4 months for the gemcitabine arm, $p = 0.014$). PFS was also significantly better in the subgroup with good Karnofsky PS who received combination chemotherapy compared to those treated with single-agent gemcitabine (HR 0.69, 95% CI 0.50–0.95, $p = 0.022$). There were similar toxicity rates seen in the two arms, with neutropenia being the most common grade 3/4 adverse event. The authors therefore recommended that this combination could be used in advanced pancreatic cancer patients with good performance status. This was, however, a post hoc analysis and the study was not sufficiently powered to show a small benefit from combination chemotherapy over gemcitabine alone.

The second phase III trial, undertaken by the UK National Cancer Research Institute (NCRI), used higher total doses of gemcitabine and capecitabine with good effect [19]. A total of 533 patients were randomized to receive gemcitabine monotherapy or GemCap. A higher rate of grade 3/4 neutropenia was observed in the combination arm, but otherwise toxicity rates and quality of life data were similar between the two arms. This study showed that GemCap was superior over single-agent gemcitabine in terms of objective response rate (19.1% vs. 12.4%, $p = 0.034$) and PFS (5.3 vs. 3.8 months, HR 0.78, 95% CI 0.66–0.93, $p = 0.004$), but the OS difference did not reach statistical significance (median 7.1 vs. 6.2 months, HR 0.86, 95% CI 0.72–1.02, $p = 0.080$). However, the investigators undertook a meta-analysis of the NCRI trial combined with the Swiss trial and the randomized phase II data published by Scheithauer and colleagues and found an overall survival benefit in favor of GemCap in the 468 patients included in the analysis (HR 0.86, 95% CI 0.75–0.98, $p = 0.02$), suggesting that most of the studies to date have been individually underpowered to detect the small benefit in OS from the addition of capecitabine [19]. The GemCap regimen remains a standard first-line regimen in the United Kingdom based on these data.

Gemcitabine and S-1

S-1 is a new oral fluorinated pyrimidine which has been used increasingly in the Far East, especially in gastric cancer. The compound consists of tegafur (a prodrug of 5-FU, converted through a multistage process in vivo) (Fig. 2), 5-chloro-2,4-dihydroxypyridine (CDHP or gimeracil), and potassium oxonate (Oxo), an inhibitor of pyrimidine phosphoribosyl transferase enzyme preferentially taken up by gastrointestinal cells, which decreases activation of 5-FU, thus theoretically reducing the

gastrointestinal side effects normally associated with 5-FU (Fig. 3). S-1 has also been developed to increase the efficacy of 5-FU by incorporating CDHP, an inhibitor of dihydropyrimidine dehydrogenase (DPD) which degrades 5-FU. Studies with S-1 in gastric and colorectal cancers suggest that there may be greater activity in genotypes from the Far East than in Western populations. Given the preclinical

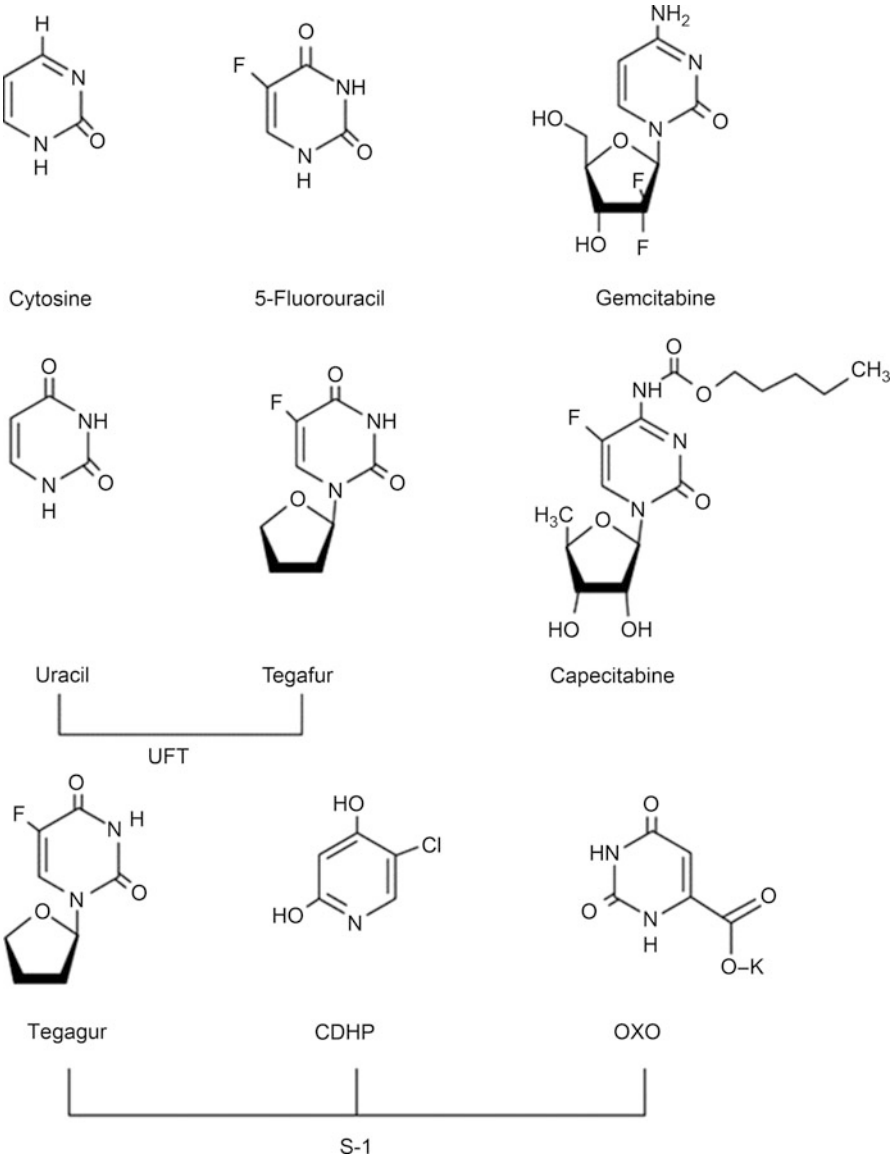

Fig. 3 Chemical structures of natural pyrimidines and synthetic analogues

data suggesting synergism between gemcitabine and 5-FU, S-1 has been investigated as an alternative in this doublet regimen.

Initial studies with single-agent S-1 in advanced pancreatic cancer have shown promising activity. A small phase II Japanese trial using the gemcitabine plus S-1 combination in 33 chemotherapy-naïve patients used a regime of S-1 orally (30 mg/m^2) twice daily for 14 consecutive days and gemcitabine (1,000 mg/m^2) on days 8 and 15, every 21 days. The reported response rate and median survival were 48% and 12.5 months, respectively, which was particularly impressive since all patients had distant metastatic disease. Neutropenia was the most prevalent grade 3/4 toxicity seen with this combination and, at 55%, appeared significantly higher than in the other gemcitabine/fluoropyrimidine combination trials.

Two multicenter phase II studies ($n = 38$ and $n = 55$), using a higher dose S-1 regimen (S-1 orally 40 mg/m^2 twice daily for 14 consecutive days and gemcitabine 1,000 mg/m^2 on days 1 and 8, every 21 days), have reported objective response rates of 32% and 44%, median PFS of 5.4 and 5.9 months, and median OS of 8.4 and 10.1 months, respectively. In both studies, neutropenia was again the most prevalent grade 3/4 toxicity. While in the study by Oh et al., the incidence of grade 3/4 neutropenia was 39.5%, in the study by Ueno et al., this was unacceptably high at 80%.

More recently, a large randomized three-arm phase III trial from Japan and Taiwan compared single-agent S-1 (80, 100, or 120 mg/day according to body surface area on days 1–28 of a 42-day cycle) and S-1 plus gemcitabine (60, 80, or 100 mg/day of S-1 according to body surface area on days 1–14 and gemcitabine 1,000 mg/m^2 on days 1 and 8 of a 21-day cycle) with gemcitabine alone (gemcitabine 1,000 mg/m2 on days 1, 8, and 15 of a 28-day cycle) [20]. The objective of this study was to demonstrate non-inferiority of S-1 as well as superiority of S-1 plus gemcitabine compared with standard single-agent gemcitabine. The primary endpoint was OS. A total of 832 chemotherapy-naïve patients with ECOG PS 0–1 were enrolled. While non-inferiority of S-1 to gemcitabine was shown (median OS 9.7 vs. 8.8 months HR 0.96, 97.5% CI 0.78–1.18, $p < 0.001$), the study failed to demonstrate the superiority of S-1 plus gemcitabine to gemcitabine alone (median OS 10.1 vs. 8.8 months, HR 0.88, 97.5% CI 0.7–1.08, $p = 0.15$). However, it is interesting to note that the combination of S-1 plus gemcitabine was associated with a statistically significant improvement in both objective response rate (29.3% vs.13.3%, $p < 0.001$) and median PFS (5.7 vs. 4.1 months, HR 0.66, 97.5% CI 0.54–0.81, $p < 0.001$) compared with single-agent gemcitabine. In contrast to previous phase II trials, lower doses of S-1 were used in the combination arm to minimize the risk of neutropenia. However, the rate of grade 3/4 neutropenia was 62.2% in the S-1 plus gemcitabine arm compared to 41.0% ($p < 0.001$) and 8.8% ($p < 0.001$) in the gemcitabine arm and S-1 arm, respectively. Increased rates of grade 3/4 thrombocytopenia, diarrhea, vomiting, and stomatitis were also reported in the combination treatment group.

Extrapolation of these results to the general treatment population is not recommended, considering the differences seen in results between Japanese and

Western population sub-analyses in earlier studies with S-1. It would be certainly interesting to determine the activity of S-1 in a Western pancreatic cancer population, to see if the discrepancies between East and West seen in gastric and colorectal cancer patients are also true in this disease setting (Table 3).

Gemcitabine and UFT

UFT, or uracil-tegafur, is another oral fluoropyrimidine (Figs. 2 and 3) which is a combination of 1-(2-tetrahydrofuryl)-5-fluorouracil (tegafur, a prodrug of 5-FU) and uracil (which inhibits catabolism of 5-FU, thus increasing the plasma concentration). Single-agent UFT showed no statistically significant activity in advanced pancreatic cancer. However, several phase II studies have been conducted combining gemcitabine with UFT, which have shown moderate activity. Currently, no phase III data have been published, but data from the phase II studies using FDR gemcitabine dosing or use of leucovorin modulation suggest modest activity. The toxicity profile appears similar to other oral fluoropyrimidine-containing gemcitabine combinations and again is more convenient for patients than infused 5-FU. However, until further supporting data are available, the gemcitabine-UFT combination cannot be recommended as a standard first-line treatment.

Gemcitabine and Platinum Agents

Preclinical data suggest that the combination of gemcitabine with platinum analogues not only increases platinum-induced DNA cross-links but also effectively inhibits their repair. Cisplatin also appears to enhance the incorporation of gemcitabine triphosphates into DNA and induces apoptosis of tumor cells. Synergistic cytotoxicity has been observed in vitro and relates to multiple mechanisms of drug interaction between the two agents. Based on these observations, clinical studies were initiated to investigate the efficacy of this combination in advanced pancreatic cancer.

Single-agent cisplatin (also known as cis-diamminedicholoroplatinum or CDDP) had been previously shown to have useful activity in advanced pancreatic cancer in a small phase II study of 33 patients. A response rate of 21% was reported by the authors, using a dose of 100 mg/m^2 on a 4-weekly cycle, but required intensive hydration to prevent nephrotoxicity, which usually necessitated an overnight inpatient admission. Other toxicities seen with cisplatin include neurotoxicity, ototoxicity, alopecia, myelosuppression, and nausea and vomiting. The addition of cisplatin to gemcitabine is logical, as there are no overlapping, dose-limiting toxicities. Several phase II studies have combined gemcitabine with cisplatin in different regimens, providing median OS rates of 5.6–9.6 months and response rates of 9–31%. The major reported grade 3/4 toxicity was myelosuppression (as high as 93% in one study), with the least toxic regimen also reporting the lowest efficacy.

The first randomized phase III study of gemcitabine with or without cisplatin recruited 107 patients with both locally advanced (approximately 50%) and metastatic disease [10]. The median OS showed a non-statistically significant improvement with the addition of cisplatin (6.9 months for the combination arm compared to 4.6 months for the gemcitabine alone arm, $p = 0.48$). However, the median TTP was

Table 3 Phase II and III trials of gemcitabine and S-1 or UFT in advanced pancreatic cancer

Study	Treatment	No. of patients	Median OS (months)	Median TTP (months)	Response rate (%)
Nakamura et al. (2006)	Gemcitabine 1,000 mg/m^2 on d8 and d15 with S-1 30 mg/m^2 twice daily for 14 days, every 21 days	33	12.5	5.4	48
Oh et al. (2010)	Gemcitabine 1,000 mg/m^2 on d1 and d8 with S-1 40 mg/m^2 twice daily for 14 days, every 21 days	38	8.4	5.4[a]	32
Ueno et al. (2011)	Gemcitabine 1,000 mg/m^2 on d1 and d8 with S-1 40 mg/m^2 twice daily for 14 days, every 21 days	55	10.1	5.9[a]	44
Ueno et al. (2013) [20]	Gemcitabine 1,000 mg/m2 on d1, d8, and d15, every 28 days	832	8.8	4.1[a]	13.3
	S-1 80, 100, or 120 mg/day (according to BSA) for 28 days, every 42 days		9.7	3.8[a]	21.0
	Gemcitabine 1,000 mg/m2 on d1, d8, and S-1 60, 80, or 100 mg/day (according to BSA) for 14 days, every 421 days		10.1	5.7[a]	29.3
Lee et al. (2004)	Gemcitabine 1,000 mg/m^2 on d1, d8 and d15 with UFT 390 mg/m^2/day in three divided doses from d1 to d14, repeated every 28 days	22	5.8	4.2	22.7
Kim et al. (2002)	FDR gemcitabine 800 mg/m^2 d1, d8, and d15 with UFT 200 mg/m^2/day on d1–d21 and oral leucovorin 90 mg/day d1–d21, repeated every 28 days	30	7.2	3.0	17
Feliu et al. (2000)	Gemcitabine 1,000 mg/m^2 on d1, d8, and d15 with UFT, IV leucovorin 250 mg/m^2 on d1, and oral leucovorin d2–d14, with UFT 390 mg/m^2/day in two divided doses on d1–d14, repeated every 28 days	42	7	4	16
Feliu et al. (2002)	FDR gemcitabine 1,200 mg/m2 on d1, d8, and d15, with UFT 400 mg/m^2/day in two to three divided doses d1–d21, repeated every 28 days	43	11	6	33

[a]Progression-free survival

statistically significantly increased in favor of the combination arm at 4.6 months versus 1.8 months for the monotherapy arm ($p = 0.048$). A similar benefit was seen in the response rate, which was 26.4% versus 9.2%, in the combination and monotherapy arm, respectively ($p = 0.02$). An alternative gemcitabine dosing schedule was employed in a second randomized phase III study, which recruited 195 chemotherapy-naïve patients with both metastatic and locally advanced disease not amenable to surgery at diagnosis [13]. In contrast to the previous study, approximately 80% of patients in each arm had metastatic disease. The study again demonstrated no statistically significant advantage in median OS in the combination arm compared to gemcitabine alone (7.5 months vs. 6.0 months, $p = 0.15$), and response rates were comparable between the two groups (10.2% vs. 8.2%). However, the proportion of patients with stable disease was statistically significant higher in the doublet regimen arm (60.2% versus 40.2%, $p < 0.001$). The gemcitabine/cisplatin combination also conferred an increased but statistically nonsignificant PFS benefit of 5.3 months compared to 3.1 months ($p = 0.053$). In a post hoc analysis of patients with Karnofsky PS (KPS) of 90–100%, the gemcitabine/cisplatin regimen resulted in an increase in median OS from 6.9 to 10.7 months ($p = 0.051$), while no significant difference was seen in patients with KPS of 70–80% (4.9 months vs. 4.8 months, respectively). PFS was also superior in patients with KPS 90–100% receiving the combination (7.7 months vs. 2.8 months, $p = 0.013$), whereas no advantage from the addition of cisplatin to gemcitabine was seen in the remaining patients. A subgroup analysis also demonstrated that in the patients with metastatic disease, median OS was 7.2 months versus 4.7 months for the combination and monotherapy arms, respectively. The grade 3/4 toxicity rates were similar between the two arms, with only nausea and vomiting being more frequent in the combination arm (22.2% vs. 5.9%, $p = 0.0002$). When patient quality of life (QOL) was assessed using the Spitzer QOL index, no difference was observed between the two groups.

More recently, the results of a larger ($n = 400$) randomized phase III trial (GIP-1) have been reported where a weekly schedule of cisplatin plus gemcitabine was compared with standard single-agent gemcitabine in patients with locally advanced or metastatic disease [21]. Similar to previous studies, no difference was observed in OS (primary endpoint). Patients who received combination treatment had a median OS of 7.2 months compared with 8.3 months for those who were treated with gemcitabine alone (HR 1.10, 95% CI 0.89–1.35, $p = 0.38$). Median PFS (3.8 vs. 3.9 months, HR 0.97; 95% CI 0.80–1.19, $p = 0.80$) and objective response rate (12.9% vs. 10.1%, $p = 0.37$) were also similar between arms. On the other hand, an increased risk of grade 3/4 hematologic toxicities (including neutropenia, anemia, and thrombocytopenia) was observed in the investigational treatment group.

Oxaliplatin (Eloxatin™) has been shown to be non-inferior to cisplatin in the treatment of advanced gastric cancer. Oxaliplatin is more convenient than cisplatin as it does not require prehydration, meaning that treatment can be administered on an

outpatient basis. The main toxicity is peripheral sensory neuropathy, which tends to occur with cumulative exposure. The other notable side effect experienced by patients is laryngeal dysesthesia. Oxaliplatin monotherapy has shown disappointing activity in advanced pancreatic cancer, but one phase II study of 47 patients found that in combination with gemcitabine, it provided an OS of 6.2 months and response rate of 10.9%. As previously discussed, promising OS results were reported in a phase II study using FDR gemcitabine with oxaliplatin (GEMOX) in 64 patients with advanced pancreatic cancer (OS 9.2 months, response rate 30.6%), but the subsequent phase III evaluation failed to confirm a statistically significant difference compared to gemcitabine monotherapy [16]. The French Multidisciplinary Clinical Research Group (GERCOR)/Italian Group for the Study of Gastrointestinal Tract Cancer (GISCAD) intergroup phase III study also compared gemcitabine to the GEMOX regimen [12]. Median OS was increased with the doublet regimen, but again, the difference was not statistically significant (9.0 months vs. 7.1 months, $p = 0.13$), therefore failing to meet the trial primary endpoint. A subgroup analysis of patients with metastatic cancer also demonstrated a non-statistically significant OS advantage with the doublet regimen (8.5 months vs. 6.7 months, $p = 0.17$), whereas response rate, PFS, and clinical benefit response were all statistically significantly superior in the combination arm (26.8% vs. 17.3%, $p = 0.04$; 5.8 months vs. 3.7 months, $p = 0.04$; 38.2% vs. 26.9%, $p = 0.03$, respectively). Both regimens were well tolerated, although an increased rate of grade 3/4 thrombocytopenia (14.0% vs. 3.2%), vomiting (8.9% vs. 3.2%), and peripheral neuropathy (19.1% vs. 0%) was observed in the combination arm.

Of interest, a pooled analysis of the German multicenter study of gemcitabine versus gemcitabine/cisplatin [19] and the GERCOR/GISCAD study of gemcitabine versus gemcitabine/oxaliplatin [18] demonstrated that gemcitabine in combination with a platinum analogue was significantly more efficacious compared to single-agent gemcitabine [22]. A total of 503 patients were evaluated in the analysis, 251 patients in the gemcitabine alone analysis and 252 in the combination group. The combination provided significant improvements in overall response rate (22% vs. 14%; HR 1.69, 95% CI 1.06–2.70, $p = 0.028$), OS (36 weeks vs. 29 weeks; HR 0.81, 95% CI 0.67–0.98, $p = 0.031$), and PFS (24 weeks vs. 14 weeks; HR 0.75, 95% CI 0.61–0.90, $p = 0.0030$) compared to gemcitabine monotherapy. These improvements were most marked in patients with metastatic disease and in patients with better initial performance status.

Two other, larger meta-analyses incorporating outcome data from 4,465 to 6,296 patients, respectively, have demonstrated statistically significant improvement in PFS and response rate with a gemcitabine and platinum combination over single-agent gemcitabine [23, 24]. Similar conclusions were also reached in a third meta-analysis, which reported that gemcitabine plus platinum provided an OS advantage over gemcitabine alone, based on analysis of three randomized phase III trials with a total of 1,077 patients (HR 0.85, 95% CI 0.74–0.96) [25]. These meta-analyses again indicate that the studies reported have been underpowered to discern any small advantage over gemcitabine alone but that gemcitabine-platinum combination,

especially gemcitabine with oxaliplatin, may provide an alternative regimen in the first-line setting.

Gemcitabine and Topoisomerase Inhibitors

Irinotecan (CPT-11, Camptosar™) is the most widely used topoisomerase inhibitor in gastrointestinal oncology. Initial studies with single-agent treatment in advanced pancreatic cancer have shown a response rate of 9%. Addition of gemcitabine in the phase II setting has increased this to 20%, and the investigators reported a 1-year survival of 27%, but only modest median TTP (2.8 months) and median OS (5.7 months) [26]. A randomized phase III study of gemcitabine with or without irinotecan ($n = 360$) reported a slightly lower OS in the combination chemotherapy arm (6.3 months vs. 6.6 months) despite a significantly better response rate (16.1% vs. 4.4%, $p = 0.001$) [27]. The most commonly reported grade 3/4 toxicity was neutropenia, which was similar between the two arms (37.6% and 32%), with only grade 3/4 diarrhea being notably higher in the combination arm (18.5% vs. 1.8%) and no reduction in patient QOL despite this. As the primary endpoint of the trial was a 40% increase in median OS, the trial was negative and this combination, although apparently active, cannot be recommended in this setting.

Exatecan (DX-8951f) is another topoisomerase inhibitor which has been investigated as a treatment for advanced pancreatic cancer. Single-agent activity in this setting is modest, and subsequent combination with gemcitabine has been reported as non-superior to gemcitabine alone in a randomized phase III study [28]. The median OS was 6.7 months in the combination arm versus 6.2 months in the monotherapy arm ($p = 0.52$), while the median TTP was 3.7 months and 3.8 months, respectively ($p = 0.22$). Also, significantly more patients in the combination arm developed grade 3/4 toxicities, especially neutropenia, thrombocytopenia, and vomiting; therefore, again, this combination cannot be recommended.

An oral topoisomerase inhibitor, rubitecan (9-nitrocamptothecin or RFS-2000), has also been evaluated in the setting of chemorefractory pancreatic cancer. While preliminary studies suggested single-agent activity, subsequent phase III trials failed to show significant survival improvement in OS compared with standard therapy [29].

Gemcitabine and Taxanes

Taxanes are diterpenes which promote the intracellular assembly of microtubules and inhibit the depolymerization of tubulin, causing cell cycle arrest in the G2/M phase. Paclitaxel (Taxol™) was derived from the Pacific yew tree (*Taxus brevifolia*) and has been in clinical use in ovarian, breast, and lung cancer. Docetaxel (Taxotere™) is a semisynthetic taxane derived from an inactive precursor extracted from the European yew tree (*Taxus baccata*) and has been used in a wide variety of cancers. Preclinical data of docetaxel shows activity in pancreatic cancer cell lines, and phase II trials with single-agent docetaxel (dose ranging from 60 to 100 mg/m^2 given 3/4 weekly) in pancreatic cancer have shown modest activity (5–15% response rate and 5.9–8.3 months median OS). The most common toxicity reported in these studies was myelosuppression (grade 3/4 neutropenia ranging from 12% to 95%),

but another significant toxicity was gastrointestinal disturbance. Of note, the study which reported the lowest neutropenia rate used granulocyte colony-stimulating factor (G-CSF) support during chemotherapy.

In order to improve outcome and reduce the high rates of grade 3/4 toxicities seen with high dose single agent docetaxel, investigators have introduced docetaxel-based doublet regimens. Phase I/II studies combining docetaxel with gemcitabine have reported response rates from 12.5% to 18%, but median OS of 4.7–8.9 months, no better than docetaxel single-agent regimens. Grade 3/4 neutropenia was still the most commonly reported toxicity, with rates ranging from 14% to 85%, again with the lowest rates in the studies that utilized G-CSF supportive therapy. Overall, docetaxel is an effective addition to the armory of drugs for advanced pancreatic cancer, but is hindered by the high rates of grade 3/4 toxicities which may limit its clinical use and therefore cannot be currently recommended as a standard therapy.

Single-agent paclitaxel has shown some modest activity in advanced pancreatic cancer; the Southwest Oncology Group (SWOG) published data from a phase II trial reporting an overall response rate of 8%, similar to results from single-agent gemcitabine and 5-FU. Notably, the dose-intense paclitaxel regimen (250 mg/m^2 3 weekly) used in the study was with G-CSF support and resulted in 85% of patients reporting fatigue and 74% reporting nausea, vomiting, or anorexia, suggesting toxicity outweighs any clinical benefit.

Data from a phase II study using a novel micellar formulation of paclitaxel in a low molecular weight biodegradable synthetic polymer suggest similar outcome to single-agent gemcitabine (overall response rate 6.7%, median PFS 2.8 months, and median OS 6.2 months), but a more favorable safety profile [30]. More recently, a novel formulation of paclitaxel embedded in cationic liposomes (EndoTAG®-1) has been tested in a four-arm, randomized phase II trial where three different dosages of this agent were given in combination with gemcitabine versus single-agent gemcitabine [31]. A total of 212 chemotherapy-naïve patients were included. While no difference in objective response rate was observed between treatment groups, patients who were allocated to the combination arm appeared to have better median PFS (4.1–4.6 vs. 2.7 months) and median OS (8.1–9.3 vs. 6.8 months) compared with patient receiving gemcitabine alone. Furthermore, safety of the combination treatment appeared manageable with no report of treatment-related neuropathy. Overall, these data support the contention that, due to a higher therapeutic index compared with docetaxel and paclitaxel, novel formulations of taxanes can be safely combined with other cytotoxic agents for advanced pancreatic cancer.

Gemcitabine and Nab-Paclitaxel

Nab-paclitaxel (ABI-007) is a novel formulation of paclitaxel consisting of a colloidal suspension of 130-nm albumin-bound paclitaxel particles. The albumin-bound technology allows intravenous administration of paclitaxel without oil-based solvents (i.e., polyethylated castor oil and ethanol) which are normally required to solubilize this hydrophobic agent and may also cause infusion-related reactions. As a result, *nab*-paclitaxel does not need premedication and can be delivered at higher

doses and with shorter infusion schedules compared to the standard formulation of paclitaxel.

Historically, most of the available data on *nab*-paclitaxel are from studies in advanced breast cancer where this agent was proven to be more effective (in terms of response rate and TTP) compared to Cremophor-based paclitaxel. The difference in antitumor activity is thought to be secondary to a more favorable biodistribution and increased drug penetration into the tumor area of the albumin-bound formulation [32]. It has been proposed that the mechanism leading to accumulation of *nab*-paclitaxel into the tumor tissue includes transport of albumin into the interstitial space through glycoprotein-60-mediated endothelial cell transcytosis and subsequent binding of albumin to secreted protein acidic and rich in cysteine (SPARC), a glycoprotein expressed in the tumor microenvironment of most pancreatic tumors [32]. As far as safety is concerned, the toxicity profile of *nab*-paclitaxel appears to differ to that of standard paclitaxel due to a reduced risk of grade $\geq$3 neutropenia and increased risk of grade $\geq$3 peripheral neuropathy.

Using the natural vehicle properties of albumin to ensure increased drug penetration into the tumor area is an attractive strategy for tumors like pancreatic cancer which are characterized by a thick desmoplastic stroma. Further rationale for investigating *nab*-paclitaxel in this disease is provided by the results of preclinical studies where a synergistic activity with gemcitabine was observed, this possibly explained by reduced activity of the enzyme cytidine deaminase and increased intratumoral concentration of gemcitabine [33].

Following the promising results of a pivotal phase I/II trial (i.e., objective response rate 48%, median PFS 7.9 months, median OS 12.2 months among patients treated at the maximum tolerated dose) [34], the combination of *nab*-paclitaxel and gemcitabine was tested in the MPACT study, an international, multicenter, randomized phase III trial [35]. In this study 861 patients who had a Karnofsky score $\geq$70 and were chemotherapy-naïve (including adjuvant chemotherapy) were randomly allocated in a 1:1 ratio to single-agent gemcitabine (1,000 mg/mg^2 on days 1, 8, and 15, every 28 days) or gemcitabine plus *nab*-paclitaxel (gemcitabine 1,000 mg/mg^2 followed by nab-paclitaxel 125 mg/m^2 on days 1, 8, and 15, every 28 days). The primary endpoint of the study was OS. Adding *nab*-paclitaxel to gemcitabine led to a statistically significant improvement in median OS (8.5 versus 6.7 months, HR 0.72, $p < 0.0001$), median PFS (5.5 versus 3.7 months, HR 0.69, $p < 0.0001$), and objective response rate (23% versus 7%, $p < 0.001$). The proportion of patients alive at 1, 2, and 3 years in the combination treatment group and standard treatment group was 35% versus 22%, 10% versus 5%, and 4% versus 0%, respectively [36]. Neutropenia (38% vs. 27%), leukopenia (31% vs. 16%), fatigue (17% vs. 7%), and peripheral neuropathy (17% vs. 1%) were the treatment-related grade $\geq$3 toxicities which were reported significantly more frequently in the investigational arm than in the comparator arm. It is worth noting that, although *nab*-paclitaxel-related peripheral neuropathy (any grade) occurred in 54% of study patients and led to dose reduction and treatment discontinuation in 10% and 8% of patients, respectively, this appeared to be rapidly reversible (i.e., in less than 1 month) in most cases [37].

Activity and safety of this combination regimen have also been confirmed by studies conducted in Asian populations. In two small phase I/II trials which included only Chinese ($n = 21$) and Japanese ($n = 34$) chemotherapy-naïve patients, the administration of gemcitabine and *nab*-paclitaxel according to the same dose and schedule used in the MPACT trial was associated with objective response rates of 42–58%, median PFS of 5.2–6.5 months, and a median OS of 12.2–13.5 months.

Recently, alternative administration schedules of gemcitabine plus *nab*-paclitaxel have been investigated in the attempt to reduce toxicities and maintain efficacy. Administering gemcitabine and *nab*-paclitaxel once every 2 weeks (instead of weekly for 3 out of 4 weeks) has been reported to be effective and associated with a reduced risk of grade $\geq$3 neutropenia, fatigue, and neuropathy as well as reduced costs [38].

There are currently no established biomarkers to identify patients who are more likely to benefit from the use of *nab*-paclitaxel. Based on the putative role of SPARC in the mechanisms of penetration of nab-paclitaxel into the tumor area, it was originally hypothesized that expression of this glycoprotein could serve as a valuable tool for patient selection. However, retrospective analyses of the MPACT trial showed lack of association between clinical benefit from *nab*-paclitaxel and expression of SPARC either in the tumor tissue (stroma or epithelia) or in the plasma.

Gemcitabine with Other Agents

Gemcitabine has been combined with several additional chemotherapy agents. Pemetrexed (Alimta™, MTA, LY231514) is a pyrrolopyrimidine-based antifolate compound routinely used in non small cell lung cancer. Single-agent use in advanced pancreatic cancer shows minimal activity. However, preclinical studies have shown a synergistic effect with gemcitabine, suggesting improved clinical activity. The potential for this combination was seen with a reported response rate of 32% in the phase II setting. Unfortunately, this combination did not improve OS, PFS, time to treatment failure, or 1-year survival in a randomized phase III study (OS 6.2 months for the combination vs. 6.3 months, $p = 0.848$) [39]. QOL assessed by the EORTC QLQ C30 questionnaire was not statistically different between the two groups, despite a statistically greater rate of grade 3/4 myelotoxicity.

Gemcitabine has also been combined with agents previously shown to enhance the effect of cytotoxic therapy. One such agent is celecoxib, a selective cycloxygenase-2 (COX-2) inhibitor. Previous molecular studies had demonstrated an overexpression of COX-2 in pancreatic cancer cell lines, involved in inflammation, carcinogenesis, and modulation of angiogenesis. Limited phase II data are available, reporting mixed results, although any gains were modest.

Other Doublet Regimens

Doublet regimens not based on gemcitabine have also been examined. Preclinical data suggest synergistic benefit with taxanes and irinotecan. Docetaxel combined

with irinotecan in a phase II study has shown a median OS of 8.5 months, but grade 3/4 neutropenia rates of 78%, using a regimen of 60 mg/m^2 for docetaxel and 250 mg/m^2 for irinotecan on a 3-weekly cycle [40]. A weekly dosing schedule for the first 4 consecutive weeks out of every 5 weeks (35 mg/m^2 of docetaxel and 50 mg/m^2 of irinotecan) was also investigated in a phase II study [41]. The authors reported a median OS of 9.4 months in the 37 patients enrolled and a response rate of 27%. The level of grade 3/4 neutropenia reported was 30%, with a 21% rate of grade 3/4 diarrhea. This split-dosing regimen of docetaxel appears more promising, reducing the incidence of grade 3/4 toxicities, as has been demonstrated in other tumor types.

The combination of 5-FU with oxaliplatin is a standard treatment for colorectal cancer, both in the adjuvant and metastatic settings. Preclinical data suggest a synergistic effect, and the safety profile of this combination is acceptable, with myelosuppression and cumulative neurotoxicity being the predominant toxicities. A phase II study randomized 65 patients from ten French centers to receive either single-agent oxaliplatin (130 mg/m^2 3 weekly), single-agent infused 5-FU (1,000 mg/m^2 over days 1–4, every 3 weeks), or the two drugs combined [42]. The authors reported a response rate of 10% and a median OS of 9 months in the combination arm, compared to 3.4 months and 2.4 months in the oxaliplatin and 5-FU arms, respectively. Grade 3/4 neutropenia was reported in 18% and grade 3 neuropathy in 6.5% of patients in the combination arm.

More recently, a phase II study investigating 5-FU, folinic acid, and oxaliplatin (FOLFOX-6) in previously untreated advanced pancreatic cancer reported a response rate and OS of 27.6% and 7.5 months, respectively, in the 30 patients recruited [43]. Again, the regimen was well tolerated with acceptable levels of grade 3/4 toxicities. However, this regimen has not been evaluated in a phase III randomized controlled trial, and as the outcomes do not appear to be superior to those reported in other trials of gemcitabine monotherapy or gemcitabine-based combinations, this too, cannot be considered a standard regimen.

Three or More Drug Regimens

Triplet chemotherapy regimens are standard practice in the treatment of gastric and esophageal carcinomas and more recently emerged as a treatment option for colorectal cancer. The data from the doublet regimen clinical trials in advanced pancreatic cancer have only shown a modest benefit over single-agent gemcitabine at best, and therefore focus has shifted to improving combinations with triplet regimens to attempt to maximize benefit from chemotherapy.

Gemcitabine in combination with docetaxel and capecitabine (GTX) has been investigated in the United States. In a retrospective study of 35 patients (including chemotherapy-naïve and refractory patients), an overall response rate of 29% was reported, with 20% being alive at 2 years. One cycle of GTX was given over 14 days consisting of capecitabine, 750 mg/m^2 twice daily on days 1–14 (total 1,500 mg/m^2/day), with gemcitabine (750 mg/m^2) followed by docetaxel (30 mg/m^2 over) on days

4 and 11. More recently, similar results were reported in a multicenter retrospective analysis of 154 patients with locally advanced or metastatic tumors. The overall response rate was 11% and median OS was 11.6 months (25.0 months for locally advanced cancer patients and 11.3 months for patients with metastatic disease). In this study, grade $\geq$3 hematologic and non-hematologic adverse events were reported in 41% and 9% of cases, respectively.

The combination of gemcitabine, 5-FU, leucovorin, and a platinum agent has also stimulated interest, with several small studies having interval results presented at meetings, but with no study demonstrating a significant improvement in OS compared to gemcitabine alone or standard gemcitabine doublet regimens.

Gemcitabine was combined with cisplatin, epirubicin, and 5-FU in a regimen termed PEF-G. Promising results were obtained in the phase II setting, with a response rate of 58% and a median OS of 10 months. It was then compared to gemcitabine alone in a randomized phase III study which recruited 99 patients [44]. The treatment regimens used were 40 mg/m^2 cisplatin and 40 mg/m^2 epirubicin both given on day 1, 600 mg/m^2 gemcitabine administered on days 1 and 8, and fluorouracil 200 mg/m^2/day given by continuous infusion on days 1–28 of a 4-week cycle (PEF-G regimen), compared to 1,000 mg/m^2 gemcitabine given once a week for 7 of 8 consecutive weeks in cycle 1 and for 3 of 4 weeks thereafter in the gemcitabine only arm. The response rates were 38.5% for the combination arm compared to 8.5% for single-agent gemcitabine ($p = 0.0008$). Interestingly, the authors chose PFS at 4 months as the primary endpoint to reflect that the majority of patients who failed on first-line treatment usually went on to have salvage regimens within the first 4 months, thus affecting the 6-monthly median OS rates. The combination arm resulted in a PFS rate at 4 months of 60% (95% CI 46–72) versus 28% (95% CI 17–42) for the gemcitabine arm (HR 0.46, 95% CI 0.26–0.79) although toxicity was also increased; the rate of grade 3/4 neutropenia was 43% versus 14%, respectively ($p < 0.0001$). The authors conclude that PEF-G is an attractive regimen with significant activity compared to single-agent gemcitabine. However, a larger randomized trial is needed to confirm the exact impact of PEF-G on clinical outcome. More recently, in a randomized phase II trial including both locally advanced and metastatic cancer patients, the same investigators showed that replacing epirubicin (30 mg/m^2 day 1 and 15) with docetaxel (25–30 mg/m^2 day 1 and 15) in a 4-weekly regimen including cisplatin (30 mg/m^2 day 1 and 15), gemcitabine (800 mg/m^2 day 1 and 15), and capecitabine (1,250 mg/m^2/day days 1–28) was associated with a higher response rate (60% vs. 37%) and a lower incidence of grade $\geq$3 neutropenia (4% vs. 13%).

FOLFIRINOX

The combination of 5-FU with leucovorin, irinotecan, and oxaliplatin (FOLFIRINOX regime) has now become a standard first-line treatment for patients with advanced pancreatic cancer. This combination chemotherapy was originally tested in a small phase II study where 46 chemotherapy-naïve patients were treated with 2-weekly cycles of oxaliplatin 85 mg/m^2 and irinotecan 180 mg/m^2 plus leucovorin 400 mg/m^2 followed by bolus FU 400 mg/m^2 on day 1, then FU

2,400 mg/m^2 as a 46-h continuous infusion. The objective response rate was reported as 26%, while median TTP and OS were 8.2 months and 10.2 months, respectively. Grade 3/4 neutropenia rates were high at 52%, and grade 3 neuropathy was seen in 15% of patients.

More recently, a large randomized phase III trial (ACCORD-11) compared FOLFIRINOX against standard treatment with single-agent gemcitabine [45]. In this study, 342 patients with age $\leq$75 years and ECOG PS $\leq$1 were randomized in a 1:1 ratio to receive 6 months of FOLFIRINOX or gemcitabine as frontline treatment for metastatic disease. OS was the primary endpoint. The study was successful in that patients who were randomly assigned to the investigational arm were found to have better median OS (11.1 versus 6.8 months, HR 0.57, $p < 0.001$), PFS (6.4 versus 3.3 months, HR 0.47, $p < 0.001$), and objective response rates (31.6% versus 9.4%, $p < 0.001$) compared to those who received standard treatment. Safety data from this study were in line with those reported in the pivotal phase II trial. In particular, FOLFIRINOX treatment was associated with an increased risk of grade $\geq$3 neutropenia (45.7% versus 21.0%), febrile neutropenia (5.4% versus 1.2%), sensory neuropathy (9.0% versus 0%), diarrhea (12.7% versus 1.8%), and thrombocytopenia (9.1% versus 3.6%) compared to gemcitabine. However, it is also worth noting that, despite the increased toxicity, patients who were treated with FOLFIRINOX were less likely to experience a significant deterioration of quality of life at 6 months (31% versus 66%, HR 0.47, $p < 0.001$). More recently, Singhal et al. reported the results of another randomized phase III study which was conducted in India and had the same design and primary endpoint as the ACCORD-11 trial [46]. This study included 310 metastatic pancreatic cancer patients with ECOG performance status $\leq$1. Similar to the study by Conroy et al., an improvement in median OS (10.8 versus 7.4 months, HR 0.48, $p < 0.001$), median PFS (5.6 versus 3.1 months, HR 0.44, $p < 0.001$), and objective response rates (29.6% versus 8.3%, $p < 0.001$) was observed for patients treated with FOLFIRINOX compared with those treated with gemcitabine. Notably, safety and quality of life data were also very similar between the two trials, with 29% of patients in the FOLFIRINOX arm experiencing a definitive deterioration of quality of life at 6 months compared with 59% in the gemcitabine group (HR 0.45, $p < 0.001$).

While concerns were initially raised regarding the safety and feasibility of administering triplet combination chemotherapy with 5-FU, irinotecan, and oxaliplatin in real-world metastatic pancreatic cancer patients, efficacy and safety data from the abovementioned phase III trials have been largely reproduced in a number of retrospective/observational studies. This provides further support to the contention that FOLFIRINOX should be regarded as the optimal first-line treatment choice for fit patients (i.e., age $\leq$75 years and ECOG PS $\leq$1) with no significant comorbidities. Nevertheless, frequency and severity of treatment-related toxicities has prompted the investigation of modified versions of this regimen in the attempt to improve its safety profile while maintaining efficacy. In particular, data from retrospective studies suggest that omitting the bolus 5-FU and/or reducing the dose of irinotecan may minimize the risk of grade 3/4 toxicities without significantly

affecting overall treatment outcomes. Using alternative, more tolerable, schedules of FOLFIRINOX is also important considering that this regimen has been increasingly used as chemotherapy backbone for the investigation of combination strategies with novel therapeutics in clinical trials.

Second-Line Chemotherapy

The relatively high occurrence of early disease progression in advanced pancreatic cancer, despite best available current first-line therapy, has resulted in a need to define optimal second-line therapy. However, due to the generally poor performance status and unfavorable prognosis of refractory patients, conducting meaningful phase III studies has been difficult, reflected by the sparse literature reports in this setting.

Studies investigating single-agent chemotherapy (capecitabine, oxaliplatin, irinotecan, raltitrexed, and taxanes) in patients with good performance status have reported some benefit as second-line agents, with reported median PFS ranging from 1 to 4 months and median OS from 4 to 7.5 months. In order to improve on these promising but modest results, combination chemotherapy regimens or new drug formulations have been developed for second-line treatment. Over the last few years, four phase III trials have addressed the optimal management of refractory patients who experience progression after first-line gemcitabine-based therapy. Overall, the results from these trials suggest that salvage chemotherapy improves survival compared with best supportive care, and combination chemotherapy may be superior over single-agent chemotherapy.

The use of second-line treatments in pancreatic cancer has certainly increased and contributed to the improved median OS reported in recent trials. Nevertheless, there is still no international consensus on a second-line regimen. Also, the recent availability and increased use of new combination treatments (especially non-gemcitabine regimens such as FOLFIRINOX) in the first-line setting of advanced pancreatic cancer inevitably limits the generalizability of the results of previous clinical trials which were conducted in the gemcitabine era. Finally, it is worth considering that best supportive should be still considered as an option for those patients whose performance status contraindicates the use of further treatment.

Oxaliplatin-Based Regimens (OFF and FOLFOX)

The evidence that second-line chemotherapy provides a survival advantage in refractory pancreatic cancer was provided by the CONKO-003 randomized phase III trial [47, 48]. The study initially randomized patients with Karnofsky score >60% to receive either oxaliplatin, folinic acid (FA) and 24 h infused 5-FU (OFF regimen), or best supportive care. Forty-six patients were recruited out of a planned 165, before the study was discontinued due to low recruitment. At that time, a clinically significant improvement in median OS with OFF was shown (4.82 months vs.

2.30 months, HR 0.45, $p = 0.008$) with no major issues in terms of treatment-related adverse events [47]. The best supportive care arm was closed to recruitment after this analysis, and patients were instead randomized to an alternative control arm of FA and 24 h infused 5-FU (FF regimen). The primary endpoint of this study was OS and a total of 168 patients were recruited. The median OS with OFF was 5.9 months compared to 3.3 months with FF (HR 0.66, $p = 0.01$). The median PFS also statistically significantly favored OFF (2.9 months vs. 2.0 months; HR 0.68, $p = 0.019$) suggesting that oxaliplatin in combination with 5-FU/FA is an attractive treatment choice for patients of good performance status who have failed gemcitabine therapy. Of note, treatment-related adverse events did not appear to differ between treatment arms with the only exception of an increased risk of grade 1–2 (38.2% vs. 7.1%) and grade 3 (4.0% vs. 0%) toxicities for patients randomized to the OFF group [48].

Interestingly, the results of the CONKO-003 study have been recently challenged by the findings of the PANCREOX trial. In this randomized phase III trial, 108 patients with ECOG PS $\leq$2 received 5-FU and folinic acid or modified FOLFOX6 (mFOLFOX6) for the treatment of gemcitabine-refractory advanced pancreatic cancer [49]. The primary endpoint of the study was PFS and this was not different between the study arms (median PFS 2.9 months versus 3.1 months; HR 1.0, $p = 0.99$). Interestingly, median OS was inferior in patients randomly allocated to mFOLFOX6 (6.1 months versus 9.9 months, HR 1.78, $p = 0.02$). The unexpected findings of the PANCREOX study are of difficult interpretation. However, they could be explained by the higher rate of grade $\geq$3 adverse events (63% vs. 11%) and treatment discontinuation (20.4% vs. 1.9%) and a lower use of subsequent lines of treatment (6.8% vs. 25%) in the mFOLFOX6 arm compared with the 5-FU and folinic acid arm. Therefore, an oxaliplatin-based doublet regimen still remains a reasonable treatment option for patients who have progressed to a gemcitabine-based treatment and are still fit to receive further chemotherapy.

Smaller studies have also investigated oxaliplatin in combination with capecitabine (i.e., CAPOX). In a phase II study that included 39 patients who had progressed to a gemcitabine-based first-line treatment, CAPOX was associated with a response rate of 2.6%, median PFS of 9.9 weeks, median OS of 23 weeks, and a 1-year OS rate of 21%. The safety profile was overall similar to that reported for the OFF and mFOLFOX6 regimens.

Irinotecan-Based Regimens and Nanoliposomal Irinotecan

A number of small studies have shown activity of Irinotecan as a monotherapy or in combination with other cytotoxic agents in refractory pancreatic cancer patients. A phase II study, which randomized 38 patients who had failed gemcitabine first-line therapy, to either raltitrexed alone or irinotecan plus raltitrexed was closed early due to the finding of a clear benefit from the combination arm. The primary endpoint of the trial was response rate, which was noted to be 16% in the combination arm versus 0% with raltitrexed monotherapy at the first interim analysis. Despite the

higher incidence of any grade toxicities with the combination, the rate of grade 3/4 toxicities was similar in both arms. The activity of irinotecan-based treatments in the refractory setting was confirmed in a phase II study where the use of FOLFIRI (i.e., irinotecan, 5-FU, and folinic acid) was associated with a response rate of 8%, median PFS of 3.2 months, and median OS of 5 months in 50 patients who had been previously treated with gemcitabine plus a platinum agent. In a randomized phase II trial, a modified FOLFIRI regimen (i.e., mFOLFIRI.3) was compared with mFOLFOX for the treatment of gemcitabine-refractory pancreatic cancer [50]. A total of 61 patients were enrolled in this study. No difference in 6-month survival rate (primary endpoint of the study) was observed, this being 27% for the mFOLFIRI.3 arm and 30% for the mFOLFOX arm (95% CI 15–49%). Treatment groups appeared also similar in terms of disease control rate (23% vs. 17%), median PFS (8.3 vs. 6.0 weeks), and median OS (16.6 vs. 14.9 weeks). Although the overall rate of grade 3/4 toxicities was the same in both arms (38%), patients who were treated with mFOLFIRI.3 experienced more grade 3/4 diarrhea (7% vs. 0%) but less grade 3/4 asthenia (3% vs. 14%) compared with those who received mFOLFOX.

Recently, a novel liposomal formulation of this cytotoxic agent has been developed to ensure increased drug stability in the circulation and higher concentration of the active metabolite SN-38 in the tumor area. In a phase II study of 40 patients who had been previously treated with gemcitabine-based therapy, second-line treatment with single-agent nanoliposomal irinotecan was associated with an objective response rate of 7.5%, disease control rate of 50.0%, a median PFS of 2.4 months, and a median OS of 5.2 months. In a subsequent open-label, three-arm, phase III trial (NAPOLI-1), 417 pancreatic cancer patients with Karnofsky score $\geq$70 and gemcitabine-refractory tumors were randomized to nanoliposomal irinotecan as single agent or in combination with 5-FU and folinic acid or 5-FU and folinic acid alone [51]. The primary endpoint of the study was OS. While no difference in outcome was observed between single-agent nanoliposomal irinotecan and 5-FU plus folinic acid for any of the outcome measures, patients who were treated with nanoliposomal irinotecan plus 5-FU and folinic acid had better median OS (6.1 months versus 4.2 months, HR 0.67, $p = 0.012$), median PFS (3.1 months versus 1.5 months, HR 0.56, $p = 0.0001$), and objective response rate (16% versus 1%, $p = 0.0001$) compared to those who received 5-FU and folinic acid alone. The safety profile of nanoliposomal irinotecan was manageable with most common grade $\geq$3 adverse events in the combination arm including neutropenia (27%), fatigue (14%), diarrhea (13%), and vomiting (11%). Based on these results, nanoliposomal irinotecan in combination with 5-FU and folinic acid has been recently approved as a treatment option in the second-line setting of metastatic pancreatic cancer.

Other Chemotherapy Combinations

A series of different phase II studies evaluating alternative options in gemcitabine-refractory advanced disease have only reported modest improvements in clinical outcome. The majority of these combinations have been doublet regimens.

A gemcitabine and oxaliplatin doublet regimen was used in a phase II study which recruited 33 patients who had progressed on or after receiving gemcitabine as first-line therapy. The median duration of response was 4.5 months, and median survival was 6 months, which, with a reported CBR rate of 54%, suggest that this combination may warrant further investigation in a randomized phase III trial. Oxaliplatin has also been combined with raltitrexed in gemcitabine resistant cases, giving similarly promising results, particularly in patients with a previous PFS of greater than 6 months. Retrospective studies also showed similar outcomes when oxaliplatin was combined with S-1.

Other platinum agents were investigated in the refractory setting. In a randomized phase III trial comparing two sequential strategies, a response rate of 7% and a clinical benefit rate of 45% were observed in patients who received cisplatin plus 5-FU and folinic acid after progression to single-agent gemcitabine [52]. This study also showed that first-line cisplatin plus 5-FU and folinic acid followed by second-line gemcitabine was not superior in terms of overall survival compared with the opposite sequence.

A combination of capecitabine and docetaxel was investigated in a phase II trial which included 43 gemcitabine-refractory patients. The investigators reported an objective response rate of 14%, median PFS of 3.7 months, and median OS of 5.3 months. It is worth noting, however, that 50% of patients experienced grade 3/4 toxicity.

Second-line studies with single-agent chemotherapy have been conducted especially in Japan and used S-1. In two phase II trials including 40 and 21 gemcitabine-refractory patients, respectively, S-1 administered at a dose of 40 mg/m^2 twice daily for 28 days, followed by 14 days' rest was associated with response rates of 10–15%, clinical benefit rate of 53–58%, median PFS of 2.0–4.1 months, and median OS of 4.5–6.3 months. Although it is not known whether these findings can be generalized to a Western population, they suggest that single-agent treatment with S-1 may be a reasonable option in the refractory setting.

The positive results of the ACCORD-11 and MPACT trial in chemotherapy-naïve patients have recently encouraged the use of FOLFIRINOX and gemcitabine plus nab-paclitaxel as chemotherapy regimens in patients who had progressed to gemcitabine-based and FOLFIRINOX chemotherapy, respectively. Although there are currently no data from prospective clinical trials, a number of small retrospective studies have reported promising efficacy and safety data suggesting that these combination treatments may also have a potential role in the refractory setting. For example, in a prospectively recorded series of 57 advanced pancreatic cancer patients who were treated with gemcitabine plus *nab*-paclitaxel following progression to frontline FOLFIRINOX, interesting outcome data were reported with an objective response rate of 17.5%, a clinical benefit rate of 58%, and a median PFS of 5.1 months. Median OS from start of second- and first-line treatment was 8.8 months and 18.0 months, respectively. Of note, the safety profile of gemcitabine plus *nab*-paclitaxel did not differ significantly from that reported in the MPACT study. In another retrospective series of 27 patients who had progressed to gemcitabine, second-line

FOLFIRINOX was associated with a response rate of 18.5%, CBR of 63%, median TTP of 5.4 months, and median OS of 8.5 months. Again, the rate of grade $\geq$3 neutropenia (55.6%) was in line with that reported in the ACCORD-11 trial.

Careful selection of appropriate patients from those who progress after first-line therapy is crucial to deciding who will benefit from second-line treatment. Single-agent second-line chemotherapy may be associated with a lower rate of toxicities, along with a lower rate of clinical response. Therefore, combination chemotherapy should be considered for those who have a good baseline PS. As best supportive care measures become more refined, the oncologist is faced with the paradox of maximizing outcome while maintaining or improving QOL. The greater the number of chemotherapy lines or number of drugs per combination, the higher the likelihood of treatment related toxicities. As a result, emphasis has shifted over the last decade toward determining the role of targeted agents alongside chemotherapy (Tables 4 and 5).

Targeted Agents

The Epidermal Growth Factor Receptor (EGFR)

Adding novel targeted agents to chemotherapy has been a successful therapeutic strategy in a number of solid tumors. The epidermal growth factor receptor (EGFR) has been the subject of targeted therapy, using both monoclonal antibodies to the receptor itself such as cetuximab and panitumumab and small molecule receptor tyrosine kinase inhibitors (TKIs) such as erlotinib, gefitinib, and afatinib.

The human EGFR, a transmembrane glycoprotein receptor with an extracellular ligand-binding domain and an intracellular tyrosine kinase domain, is part of the ErbB family, which also includes ErbB-2 (Her-2), ErbB-3, and ErbB-4. Ligand binding to the EGFR stimulates receptor homodimerization, or heterodimerization with another receptor from the family, and results in phosphorylation of the tyrosine kinase domains and a cascade of intracellular events which lead to cell cycle progression, proliferation, and differentiation. The EGFR and two of its ligands, epidermal growth factor (EGF) and transforming growth factor alpha (TGFα), are found in normal pancreatic acini and ducts and are overexpressed in pancreatic cancers [53].

Erlotinib

Preclinical studies of erlotinib (Tarceva™), a selective small molecule inhibitor of the EGFR tyrosine kinase domain demonstrated that it can completely prevent EGF-induced autophosphorylation of head and neck cancer xenografts, inhibit in vitro proliferation of colon cancer cells that overexpress EGFR and block progression through the cell cycle at G_1 phase in both. Blockade of EGFR phosphorylation was

Table 4 Randomized trials of gemcitabine and platinum agent in advanced pancreatic cancer

Study	Phase	Treatment	No. of patients	Median OS (months)	Median TTP (months)	Response rate (%)
Colucci et al. (2002) [10]	III	Cisplatin 25 mg/m^2 + gemcitabine 1,000 mg/m^2 weekly for 7 weeks followed by 2 weeks' rest, then weekly for 3 weeks of every 4 weeks for two cycles	53	6.9 ($p = 0.48$)	4.6 ($p = 0.048$)	26.4 ($p = 0.02$)
		Gemcitabine 1,000 mg/m^2 weekly for 7 weeks followed by 2 weeks' rest, then weekly for 3 weeks of every 4 weeks for two cycles	54	4.6	1.8	9.2
Heinemann et al. (2006) [13]	III	Cisplatin 50 mg/m^2 + gemcitabine 1,000 mg/m^2 on d1 and d15, every 28 days	95	7.5 ($p = 0.15$)	5.3[a] ($p = 0.053$)	10.2
		Gemcitabine 1,000 mg/m^2 on d1, d8, and d15, every 28 days	95	6.0	3.1[a]	8.2
Louvet et al. (2005) [12]	III	FDR gemcitabine 1,000 mg/m^2 on d1 and oxaliplatin 100 mg/m^2 on d2, every 14 days	157	9.0 ($p = 0.13$)	5.8 ($p = 0.04$)	26.8 ($p = 0.04$)
		Gemcitabine 1,000 mg/m^2 (over 30 min) on d1, every 14 days	156	7.1	3.7	17.3

(*continued*)

Table 4 (continued)

Study	Phase	Treatment	No. of patients	Median OS (months)	Median TTP (months)	Response rate (%)
Poplin et al. (2009) [16]	III	FDR gemcitabine 1,000 mg/m^2 on d1 + oxaliplatin 100 mg/m^2 on d2, every 14 days	276	5.9		9
		Gemcitabine 1,000 mg/m^2 weekly for 7 weeks followed by 1-week rest, then weekly for 3 weeks of every 4 weeks	280	4.9	Not reported	5
		FDR gemcitabine 1,500 mg/m^2 weekly for 3 weeks of every 4 weeks	277	6.0		10
Colucci et al. (2010) [21]		Gemcitabine 1,000 mg/m^2 weekly for 7 weeks followed by 2 weeks' rest, then weekly for 3 weeks of every 4 weeks	199	8.3	3.9[a]	10.1
		Gemcitabine 1,000 mg/m^2 weekly for 7 weeks followed by 2 weeks' rest, then weekly for 3 weeks of every 4 weeks + Cisplatin 25 mg/m^2 weekly (expect cycle 1 day 22)	201	7.2	3.8[a]	12.9

[a]Progression-free survival

Table 5 Selected trials of second-line monotherapy and doublet chemotherapy regimens in advanced pancreatic cancer

Study	Phase	Treatment	No. of patients	Median OS (months)	Median TTP (months)	Disease control rate
Monotherapy						
Boeck et al. (2007)	II	Capecitabine 1,250 mg/m^2 twice daily for d1–d14 every 21 days	37	7.5	2.2	0% CR
						0% PR
						37% SD
Park et al. (2007)	II	Irinotecan 150 mg/m^2 d1 every 14 days	28	Not reported	4.0	0% CR
						0% PR
						14.3% SD
Androulakis et al. (2005)	II	Oxaliplatin 130 mg/m^2 d1 every 21 days	18	3.5	Not reported	0% CR
						0% PR
						16.7% SD
Combination regimens						
Pelzer et al. (2011) [47]	III	5-FU 2 g/m^2/24 h + FA 200 mg/m^2 D1, 8, 15 and 22	84	3.3	2.0[a]	Not reported
		OFF (5-FU/FA as above + oxaliplatin 85 mg/m^2 D8 and 22)	76	5.9	2.9[a]	
Demols et al. (2006)	II	FDR gemcitabine 1,000 mg/m^2 d1 + oxaliplatin 100 mg/m^2 d2, every 14 days	33	6	4.2	0% CR
						22.6% PR
						38.7% SD
Reni et al. (2006)	II	Raltitrexed 3 mg/m^2 d1 + oxaliplatin 130 mg/m^2 d1, every 21 days	41	5.2	1.8[a]	0% CR
						24% PR
						26.8% SD
Tsavaris et al. (2005)	II	Oxaliplatin 50 mg/m^2 + LV 50 mg/m^2 + FU 500 mg/m^2 d1 every 7 days	30	25 weeks	22 weeks	0% CR
						23.3% PR
						30.0% SD

(*continued*)

Table 5 (continued)

Study	Phase	Treatment	No. of patients	Median OS (months)	Median TTP (months)	Disease control rate
Oettle et al. (2011)	III	5-FU 2,000 mg/m^2 d1, d8, d15, d22 + FA 200 mg/m^2 + oxaliplatin 85 mg/m^2 d8, d22, every 42 days	23	4.82 (p= 0.008)	Not reported	Not reported
		BSC	23	2.30		
Ulrich-Pur et al. (2003)	II	Raltitrexed 3 mg/m^2 d1 every 21 days	19	4.3	4	0% CR
						0% PR
						36.8% SD
		Irinotecan 200 mg/m^2 d1 + raltitrexed 3 mg/m^2 d2 every 21 days	19	6.5	5	0% CR
						15.8% PR
						31.6% SD
Gill et al. (2014) [49]	III	Infusional 5-FU + folinic acid	54	9.9	2.9[a]	8.5% CR/PR
		mFOLFOX6	56	6.1	3.1[a]	13.2% CR/PR
Wang-Gillam et al. (2016) [51]	III	5-FU 2,000 mg/m^2 d1, d8, d15, d22 + FA 200 mg/m^2	119	4.2	1.5[a]	1% CR/PR
		Nanoliposomal irinotecan 80 mg/m^2 + 5-FU 2,400 mg/m^2 + FA 400 mg/m^2 d1 every 14 days	117	6.1 (p= 0.012)	3.1[a] (p= 0.0001)	16% CR/PR (p< 0.0001)
		Nanoliposomal irinotecan 120 mg/m^2	151	4.9	2.7[a]	6% CR/PR

[a]PFS

also observed in a study of pancreatic cancer xenografts and of clinical importance; enhancement of gemcitabine-induced apoptosis was noted. Phase I testing of erlotinib established that the drug displayed dose-dependent pharmacokinetics and did not accumulate on a continuous daily dosing schedule. The maximum tolerated dose (MTD) was 150 mg once daily orally for continuous dosing, with diarrhea and cutaneous toxicity as the dose-limiting toxicities (DLTs). A phase Ib study of dose-escalation erlotinib added to gemcitabine showed that this dose could also be achieved with chemotherapy without DLTs. Fifteen of the 26 patients included in the study had advanced pancreatic cancer and an impressive 51% 1-year survival rate was observed, prompting further investigation of this combination in pancreatic cancer. The activity of the combination was confirmed in a phase III randomized controlled trial of gemcitabine plus either erlotinib or placebo [15]. In this National Cancer Institute of Canada Clinical Trials Group (NCIC CTG) study, 569 patients with unresectable locally advanced ($n = 138$) or metastatic ($n = 431$) pancreatic cancer were randomized to receive standard dosing schedule gemcitabine with erlotinib 100–150 mg/day or placebo. The study met its primary endpoint by demonstrating a small improvement in OS in the combination therapy arm (median OS 6.24 vs. 5.91 months, HR 0.82, 95% CI 0.69–0.99) as well as a statistically significant prolongation of PFS (median 3.75 vs. 3.55 months, HR 0.77, 95%CI 0.64–0.92), despite no difference in the objective response rate (8.6% vs. 8.0%). As too few patients were treated with erlotinib at 150 mg/day, the authors recommended the lower dose (100 mg/day) for clinical practice in combination with gemcitabine. This is the first study that showed a statistically significant benefit from adding a biological agent to gemcitabine, although the incremental gain is relatively minor, and this combination has therefore not been universally accepted as the standard of care, especially where cost-effectiveness is taken into account.

Erlotinib has also been shown to be active in combination with capecitabine in gemcitabine-refractory pancreatic cancer in a small phase II trial [54]. Of the 30 patients treated with capecitabine 2,000 mg/m^2/day days 1–14 of a 21-day cycle with continuous dosing of erlotinib 150 mg/day, three patients (10%) had an objective partial response, meeting the study primary endpoint, and the median OS for all patients treated was 6.5 months. There were no grade 4 toxicities recorded but grade 3 diarrhea (17%), rash (13%), hand-foot syndrome (13%), and stomatitis (10%) were all relatively common, which could limit the regimen acceptability. As is characteristic of EGFR inhibitors, development of a papulo-pustular rash is an indication of response to erlotinib and is associated with improved survival.

More recently, a sequential strategy with first-line capecitabine plus erlotinib followed by second-line gemcitabine was compared with first-line gemcitabine plus erlotinib followed by second-line capecitabine in a randomized, non-inferiority, phase III trial that included 281 patients. The primary endpoint was time to treatment failure after second-line chemotherapy (TTF-2). Although no difference was observed in TTF-2 between arms, patients who were randomized to gemcitabine plus erlotinib had better ORR (16% vs. 5%), clinical benefit rate (51% vs. 38%), and time to treatment failure after first-line treatment (TTF-1, 3.2 vs. 2.2 months, HR 0.69, $p = 0.0034$) compared with those who were treated with

capecitabine plus erlotinib [55]. Small phase II trials have also investigated erlotinib in the first-line setting combination with combination chemotherapy (gemcitabine plus capecitabine) with or without a targeted therapy (i.e., bevacizumab) with promising results.

Erlotinib has also been investigated in addition to chemoradiation for locally advanced pancreatic cancer in phase I studies. In particular, it has been added to a number of combination chemoradiation regimens including single-agent gemcitabine, gemcitabine plus paclitaxel, single-agent capecitabine, and capecitabine plus bevacizumab. Generally, in these studies treatment was well tolerated with manageable toxicity. Also, preliminary data of efficacy were encouraging.

Gefitinib

Gefitinib (Iressa™) is also an EGFR TKI which has been similarly investigated in advanced pancreatic cancer. A phase I dose-finding trial of fixed dose gefitinib (250 mg/day) in combination with gemcitabine 1,000–1,500 mg/m^2 weekly for 3 out of every 4 weeks found hematological DLTs above 1,200 mg/m^2 gemcitabine and reported a median OS of 7.13 months for 13 patients with advanced pancreatic cancer. In a subsequent phase II study of gefitinib (250 mg daily) plus gemcitabine (1,000 mg/m2 weekly), an objective response rate was observed in six out of fifty-three patients (11.3%), while median PFS and OS were 4.1 and 7.3 months, respectively [56]. Gefitinib has also been evaluated in two small phase II studies in combination with docetaxel as second-line therapy after gemcitabine. The combination appeared ineffective as the median survival time was only 2.9 and 4.5 months. Furthermore, in one of these studies, an acceptable high rate of febrile neutropenia (27%) was reported.

Like erlotinib, gefitinib has been investigated in combination with chemoradiation in patients with locally advanced pancreatic cancer. Toxicity was acceptable but the median survival was only 7.5 months in combination with gemcitabine-based chemoradiation in a phase I study of 18 patients, which does not compare favorably to phase I data of erlotinib and chemoradiation. Combination with capecitabine-based chemoradiation was highly toxic, mainly due to diarrhea, and therefore this regimen has not been recommended for further study. Also, a combination of gefitinib plus paclitaxel-based chemoradiation was tested in a small phase I study with mainly gastrointestinal toxicity.

Cetuximab

Cetuximab (Erbitux™) is a chimeric IgG-1 monoclonal antibody to the EGFR. Early phase II results of cetuximab in combination with gemcitabine were promising, with 5/58 patients achieving a partial response and a further 26 achieving disease stabilization. However, a subsequent randomized phase II trial of 40 patients treated with gemcitabine and cisplatin, with or without cetuximab, showed no significant improvement in response rate, PFS, or OS with the addition of cetuximab. More encouraging were the results of a multicenter phase II trial combining cetuximab

with the GemOx regimen in the first-line setting; a 33% response rate was seen in the 61 evaluable patients, with a further 31% achieving stable disease.

The only randomized phase III study which investigated cetuximab in pancreatic cancer was the SWOG S0205 trial [57]. A total of 766 patients with locally advanced or metastatic disease were assigned to receive gemcitabine with or without cetuximab as first-line treatment. The primary endpoint was OS. The study failed to show any survival advantage in favor of cetuximab (median OS 5.9 months for gemcitabine alone and 6.3 months for gemcitabine plus cetuximab, HR 1.06, $p = 0.19$), and no difference was observed between arms in terms of objective response rate and median PFS. Interestingly, EGFR expression did not appear to be associated with cetuximab benefit. One explanation for the relatively poor results from adding cetuximab to chemotherapy in pancreatic cancer may be the high incidence of Kirsten ras mutations (estimated as up to 90%) [58] seen in these tumors; Kirsten ras mutations are known to confer resistance to anti-EGFR antibodies in metastatic colorectal cancer; therefore, it is possible that the same is true in this setting.

Cetuximab has been demonstrated to be beneficial in combination with radiotherapy versus radiotherapy alone in head and neck cancers. The radiosensitizing properties of this anti-EGFR monoclonal antibody have also been investigated in pancreatic cancer. In the phase II PARC trial, 68 patients with inoperable locally advanced tumors were treated with gemcitabine plus cetuximab in combination with intensity-modulated radiotherapy (IMRT). Partial response was observed in 23 cases (33.8%) and 14 patients (20.6%) became suitable for a surgical resection. Two-year OS was 20%. These results were confirmed in a subsequent phase II study where combining cetuximab with gemcitabine and radiotherapy was associated with encouraging response rate (30%) and led to surgical resection in 18/23 patients with borderline resectable tumor and 3/6 patients with unresectable tumors at baseline. In the same study, pathological complete response was observed in 8% of cases. Also, cetuximab was investigated in combination with induction GEMOX chemotherapy and sequential capecitabine-based chemoradiotherapy in a phase II study of 69 patients with locally advanced disease. In this study a median OS of 19.2 months was reported and 11.3% of patients were alive at 4 years.

Vascular Endothelial Growth Factor (VEGF)

Bevacizumab

Bevacizumab (Avastin™) is a monoclonal antibody against VEGF, a proangiogenic growth factor involved in the regulation of vascular permeability and proliferation. Bevacizumab can be safely added to gemcitabine chemotherapy. A 21% response rate (and 46% disease stabilization rate) was reported from a phase II trial of 52 patients. The median PFS was 5.4 months and OS an encouraging 8.8 months. Well-described bevacizumab-related grade 3/4 toxicities including hypertension (19%), thrombosis (13%), visceral perforation (8%), and bleeding (2%) appeared more

frequent than described in previous large studies in colorectal cancer, but this did not deter further investigation. Disappointingly, a large phase III trial of this combination failed to demonstrate a role for bevacizumab in advanced pancreatic cancer. In the CALGB 80303 study, 602 patients with untreated locally advanced or metastatic tumors were randomized to receive gemcitabine plus bevacizumab or placebo in a double-blinded trial [59]. No significant survival benefit was demonstrated from the addition of bevacizumab, with a median OS of 5.8 months in the combination arm and 5.9 months in the gemcitabine/placebo arm ($p = 0.95$). Objective response rates were also similar between arms (13% vs. 10%), and interestingly trends toward a worse median PFS were observed for patients treated with bevacizumab (2.9 vs. 3.8 months, $p = 0.07$). It is also worth noting that much lower incidences of bevacizumab-related toxicities were seen in this trial than in the phase II setting (grade 3/4 hypertension 10%, perforation 0.4%, bleeding 5%, venous thrombosis 14%).

Bevacizumab has also been investigated with combination chemotherapy regimens. In a phase II trial of 50 patients with mostly stage IV pancreatic cancer treated with GemCap plus bevacizumab, the investigators reported a 22% response rate, 5.8 months median PFS, and 9.8 months OS. In another phase II trial of GemOx plus bevacizumab which included 50 patients (34 with metastatic disease), response rate was 36%, median PFS 4.9 months, and median OS 11.9 months. However, the rate of grade 3/4 toxicity was unacceptably high at 94%.

Finally, the combination of gemcitabine plus erlotinib with or without bevacizumab was assessed in 301 metastatic pancreatic cancer patients in the randomized, placebo-controlled phase III AViTA trial [60]. Although the triplet combination significantly improved the median PFS (4.6 vs. 3.6 months, HR 0.73, 95% CI 0.61–0.86, $p = 0.0002$) and showed a trend toward a higher ORR (13.5% vs. 8.6% $p = 0.0574$), the trial failed to meet its primary endpoint of a benefit in median OS (7.1 vs. 6.0 months, HR 0.89, 95% CI 0.74–1.07, $p = 0.2087$). This, combined with the high cost associated with a regimen including two targeted agents, makes it unlikely that this will be used as a standard treatment option.

Bevacizumab has also been evaluated in combination with chemoradiation for locally advanced unresectable pancreatic cancer in phase I and II studies. While the safety profile was manageable, the efficacy data appeared overall similar to those obtained with standard chemoradiation.

Other Anti-Angiogenic Agents

Sorafenib (Nexavar™) is a VEGF receptor-2 TKI and Raf-1 kinase inhibitor with demonstrated PFS benefit in renal cell carcinomas and OS benefit in hepatocellular carcinoma. A randomized, double-blind, phase III trial of gemcitabine plus sorafenib or placebo in locally advanced or metastatic pancreatic cancer patients ($n = 102$) failed to show an improvement in median PFS (primary endpoint) (5.7 months with placebo vs. 3.8 months with sorafenib, $p = 0.902$) [61].

Similar results were reported with the combination of gemcitabine plus axitinib, an inhibitor of VEGF-R 1-3, c-KIT, and PDGFR. In a randomized phase III trial

($n = 632$), median OS (primary endpoint of the study) was similar between patients who received the investigational treatment and those who were randomized to standard gemcitabine alone (8.5 vs. 8.3 months, HR 1.014, $p = 0.5436$) [62]. More recently, a randomized, placebo-controlled, phase III study comparing gemcitabine plus or minus aflibercept (VEGF trap) was discontinued for futility when a preplanned interim analysis did not show any improvement in median OS (i.e., 7.8 months for the standard arm vs. 6.5 months for the investigational arm, HR 1.17, 95% CI 0.92–1.47, $p = 0.203$) [63] (Table 6).

Other Biological Agents

Other biological agents including (but not limited to) selumetinib and trametinib (MEK inhibitors), everolimus and temsirolimus (mTOR inhibitors), trastuzumab (HER-2 inhibitor), and bortezomib (proteasome inhibitor) have been evaluated in the phase II setting, and some, including tipifarnib (farnesyl transferase inhibitor) [64], gastrazole (CCK2/gastrin receptor antagonist) [65], marimastat (matrix metalloproteinase inhibitor) [66], ganitumab (anti-IGF-1R monoclonal antibody) [67], and masitinib (multi-tyrosine kinase inhibitor) [68], reached phase III testing. However, other than erlotinib, no biological agent has demonstrated any significant survival benefit over gemcitabine alone.

Chemoradiation

In approximately 40% of cases, pancreatic cancer presents as a locally advanced tumor that is not amenable to surgical resection. Patients with inoperable, locally advanced tumors have a better prognosis than those with disseminated metastatic disease (i.e., 5-year survival 9% vs. 2%), but the optimal management strategy remains controversial. Most trials of palliative systemic chemotherapy included patients with locally advanced and metastatic disease; therefore, single-agent gemcitabine or combination chemotherapy can be considered as standard treatment options in this setting.

Although chemoradiation is a potentially useful tool to optimize local control, much less information is available on this treatment strategy. A Cochrane meta-analysis demonstrated clearly that chemoradiation is superior to best supportive care for these patients (1-year survival 58% compared to 0%, $p = 0.001$), but concluded that there was insufficient evidence to recommend it as standard treatment for locally advanced disease [69].

Chemoradiotherapy has been investigated as either upfront treatment before systemic chemotherapy or consolidation treatment after induction systemic chemotherapy. The former strategy has been assessed by two trials with contradictory results. The FFCD-SFRO phase III trial randomized 119 patients with locally advanced unresectable pancreatic cancer to receive systemic chemotherapy with

Table 6 Phase III trials using targeted agents in advanced pancreatic cancer

Trial	Protocol treatment (number of patients)	Response rate	PFS/ months	HR (95% CI)	Significance	OS/ months	HR (95% CI)	Significance
NCI CTG phase III double-blind placebo-controlled RCT	Gemcitabine + placebo (284)	8.0%	3.55			5.91		
	Gemcitabine + erlotinib (285)	8.6%	3.75	0.77 (0.64–0.92)	$p = 0.004$	6.24	0.82 (0.69–0.99)	$p = 0.038$
SWOG S0205 phase III RCT	Gemcitabine (371)	7%	3.0	1.07 (0.93–1.24)	$p = 0.18$	5.9	1.06 (0.91–1.23)	$p = 0.19$
	Gemcitabine + cetuximab (372)	8%	3.4			6.3		
CALGB 80303 Phase III double-blind placebo-controlled trial	Gemcitabine + placebo (300)	10% (unconfirmed)	2.9	Not reported	$p = 0.075$	5.9	1.04 (0.88–1.24)	$p = 0.95$
	Gemcitabine + bevacizumab (302)	13% (unconfirmed)	3.8			5.8		
AViTA phase III double-bind, placebo-controlled RCT	Gemcitabine, erlotinib + placebo (301)	8.6%	3.6	0.73 (0.61–0.86)	$p = 0.0002$	6.0	0.89 (0.74–1.07)	$p = 0.2087$
	Gemcitabine, erlotinib + bevacizumab (306)	13.5%	4.6			7.1		
BAYPAN phase III double-blind, placebo-controlled RCT	Gemcitabine + placebo (52)	19%	5.7	Not reported	$p = 0.902$	9.2	1.27 (0.837–1.932)	$p = 0.231$
	Gemcitabine + sorafenib (52)	23%	3.8			8.0		
Gem/axitinib phase III double-blind placebo-controlled RCT	Gemcitabine + placebo (316)	2%	4.4	1.066 (0.779–1.298)	$p = 0.520$	8.3	1.014 (0.786–1.309)	$p = 0.544$
	Gemcitabine + axitinib (314)	5%	4.4			8.5		
Gem/aflibercept phase III double-blind placebo-controlled RCT	Gemcitabine + placebo (275)	Not reported	3.7	1.018 (0.828–1.253)	$p = 0.865$	7.8	1.165 (0.921–1.473)	0.203
	Gemcitabine + aflibercept (271)	Not reported	3.7			6.5		

single-agent gemcitabine or cisplatin/5-FU-based chemoradiation (60 Gy) followed by gemcitabine [70]. The study was closed early due to the unexpected finding of reduced survival in the chemoradiation arm. The median OS and 1-year survival rate were 8.6 months and 32% compared to 13 months and 53%, respectively, with systemic chemotherapy alone ($p = 0.03$). Although these findings may be explained by an increased risk of toxicity in the chemoradiation arm, a per-protocol analysis of patients who received at least 75% of the planned dose of radiotherapy showed similar results. The ECOG-4201 phase III trial compared single-agent gemcitabine versus chemoradiation (50.4 Gy) with weekly gemcitabine followed by systemic gemcitabine [71]. This study was closed early due to poor accrual. Despite increased toxicity in the chemoradiation arm, in the 74 patients who were randomized, a significant survival benefit was seen from the addition of radiation therapy, with a median survival time of 11.1 months, compared to 9.2 months in those receiving gemcitabine alone ($p = 0.017$). However, it must be noted that the survival curves for the two arms only separated after around 8 months, suggesting that only a subset of the patients with chemotherapy sensitive disease actually benefited from the addition of radiotherapy and that induction chemotherapy might be a useful strategy to select such patients for chemoradiation. In support of this, a retrospective analysis of the phase II and III GERCOR studies suggested that chemoradiation after chemotherapy may improve survival in locally advanced unresectable disease [72]. In particular, in patients who did not experience local or distant tumor progression after 3 months of chemotherapy (71% of the overall population), administration of sequential chemoradiation was associated with an improvement in median PFS (10.8 vs. 7.4 months, $p = 0.005$), median OS (15.0 vs. 11.7 months, $p = 0.0009$), and 1-year survival rate (65.3% vs. 47.5%).

Nevertheless, the contention that upfront systemic chemotherapy followed by chemoradiotherapy could be superior to systemic chemotherapy alone has been challenged by the results of the LAP07 trial [73]. In this randomized phase III trial, 442 patients with inoperable locally advanced tumors were first randomized to receive 4 months of chemotherapy with gemcitabine alone or gemcitabine plus erlotinib. If at least stable disease was achieved after induction chemotherapy, then a second randomization was performed, and patients were treated with capecitabine chemoradiation (54 Gy) or systemic chemotherapy for 2 more months (with or without maintenance erlotinib depending on the outcome of the first randomization). The primary endpoint was median OS from the first randomization. The trial was stopped prematurely when the results of a preplanned interim analysis showed no survival advantage from sequential chemoradiation. In the chemotherapy group, median OS was 16.5 months compared with 15.2 months in the chemoradiotherapy group (HR 1.03; $p = 0.83$). The only difference between treatment groups was in the pattern of tumor recurrence with patients in the chemoradiotherapy arm experiencing less locoregional failure (32% vs. 46%) but more distant metastases (60% vs. 44%) compared with those in the chemotherapy arm ($p = 0.04$).

Numerous phase II trials have investigating the addition of targeted agents to chemoradiation, but such treatments remain experimental.

Conclusions

While single-agent gemcitabine has been the only available treatment option for advanced pancreatic cancer until few years ago, the therapeutic armamentarium for this disease has been recently enriched by new, more effective combination chemotherapy regimens including FOLFIRINOX, gemcitabine plus *nab*-paclitaxel, and other gemcitabine-containing regimens. This has allowed clinicians to adopt a more selective treatment approach in routine clinical practice. A number of factors, including patient clinical condition, comorbidities, treatment goals, preference, etc., are now taken into account in the decision-making process in order to weigh pros and cons of each treatment strategy and offer patients the best treatment strategy. Evidence has also increasingly emerged to support the use of non-cross resistant second-line chemotherapy at least in patients who maintain a reasonably good performance status following progression to first-line treatment.

Nevertheless, the overall prognosis of advanced pancreatic cancer remains significantly poor (i.e., <12 months), and improved treatment strategies are urgently needed. Furthermore, there is no doubt that the treatment for this disease is still largely based on the use of cytotoxic agents, while there is no role for target therapies with the only exception of erlotinib, the survival advantage associated with this drug being however marginal. Overall, this highlights the challenges encountered in the identification and validation of valuable therapeutic targets in this setting and reflects the disappointing results of clinical trials testing novel therapeutics that show initial promise in preclinical models.

The biology of pancreatic cancer is complex especially due to the strong influence of the surrounding stroma that is now universally recognized as an important determinant of the mechanisms of tumor drug penetration, resistance to treatment, and suppression of the antitumor immune response. A number of immunotherapy strategies and drugs targeting the stroma or interfering with the interplay between tumor cells and stromal components are currently under investigation and may possibly become valid treatment options in the next future with or without standard chemotherapy.

Key Practice Points

- *First-line treatment for metastatic pancreatic cancer in patients of good performance status* (i.e., *ECOG 0-1*): Palliative chemotherapy with triplet (i.e., FOLFIRINOX) or doublet (i.e., gemcitabine plus nab-paclitaxel, gemcitabine plus erlotinib, gemcitabine plus capecitabine, or gemcitabine plus platinum) combination chemotherapy or treatment within a clinical trial, plus best supportive care.
- *First line treatment for metastatic pancreatic cancer in patients of intermediate performance status* (i.e., *ECOG 2):* Palliative chemotherapy with single-agent

chemotherapy (i.e., gemcitabine) or treatment within a clinical trial, plus best supportive care alone.

- *Treatment for metastatic pancreatic cancer in patients of poor performance status* (i.e., *ECOG >2*): Best supportive care alone.
- *First-line treatment for locally advanced unresectable pancreatic cancer*: Palliative chemotherapy with triplet (i.e., FOLFIRINOX), doublet (i.e., gemcitabine plus nab-paclitaxel, gemcitabine plus erlotinib, gemcitabine plus capecitabine, or gemcitabine plus platinum), or single agent (i.e., gemcitabine), plus best supportive care. Consideration could be given to consolidation capecitabine- or gemcitabine-based chemoradiation in patients who do not experience tumor progression after at least 3–4 months of systemic chemotherapy. Surgery should also be considered for those patients who become resectable after upfront medical treatment. Enrollment into a clinical trial is a reasonable option.
- *Second-line treatment for advanced disease*: Palliative chemotherapy with oxaliplatin (i.e., OFF or FOLFOX) or irinotecan based (i.e., nanoliposomal irinotecan plus 5-FU and folinic acid) in patients who have failed first-line gemcitabine-containing treatment, plus best supportive care. Gemcitabine-based chemotherapy in patients who have received FOLFIRINOX in the frontline setting. Treatment within a clinical trial or best supportive care remains reasonable alternative strategies.

The recommended treatment algorithm for advanced pancreatic cancer is shown in Fig. 4 (Table 7).

Published Guidelines

- National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology (NCCN Guidelines®) – Pancreatic Adenocarcinoma – Version 1.2016. Available at: https://www.nccn.org/professionals/physician_gls/pdf/pancreatic.pdf
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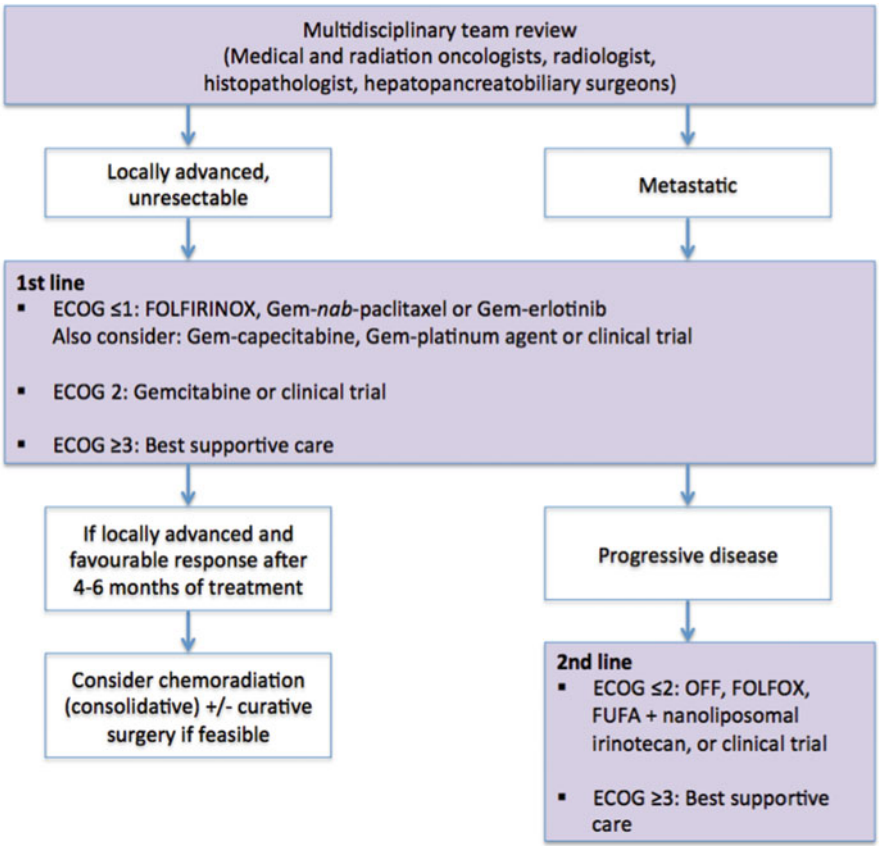

Fig. 4 Decision pathway for the management of advanced pancreatic cancer

Future Research Directions

- Treatment selection in pancreatic cancer is entirely based on clinical parameters. Better understanding of the mechanisms underlying resistance to treatment may lead to the identification of prognostic/predictive tumor biomarkers and selective use and improved efficacy of currently available treatment options.
- Less is known about the molecular aspects of carcinogenesis in pancreatic cancer compared with other tumor types. Further elucidation of the genetic basis of this disease and interaction network between tumor cells and surrounding stroma may reveal novel, potentially useful, therapeutic targets.
- Treatment of pancreatic cancer is largely based on chemotherapy and the impact of targeted therapies has been negligible. Investigation into novel treatment

Table 7 Selected current phase II and III trials in locally advanced and/or metastatic pancreatic cancer

Trial name/ sponsor	Setting	Treatment arms	Planned recruitment
NEOPAN (NCT02539537)	Locally advanced inoperable adenocarcinoma of the pancreas	FOLFIRINOX (5-FU/LV, oxaliplatin and irinotecan) or gemcitabine	170
CONKO-007 (NCT01827553)	Locally advanced inoperable adenocarcinoma of the pancreas	Induction FOLFIRINOX (5-FU/LV, oxaliplatin, and irinotecan) or gemcitabine (investigator's choice) for 12 weeks followed by either continuation of the same chemotherapy or gemcitabine-based chemoradiation	830
NCT01926197	Locally advanced inoperable adenocarcinoma of the pancreas	Modified FOLFIRINOX (5-FU/LV, oxaliplatin, and irinotecan) vs. modified FOLFIRINOX + stereotactic body radiotherapy	172
NCT02551991	Metastatic pancreatic cancer	Nanoliposomal irinotecan +5-FU/LV vs. nanoliposomal irinotecan +5-FU/LV + oxaliplatin vs. gemcitabine + *nab*-paclitaxel	168

strategies (including immunotherapy) remains key to the future management of this challenging tumor type.

Cross-References

- ▶ Circulating Tumor Cells
- ▶ Clinical Decision-Making in Pancreatic Cancer
- ▶ Development of Novel Therapeutic Response Biomarkers
- ▶ EGFR (ErbB) Signaling Pathways in Pancreatic Cancer Pathogenesis
- ▶ Emerging Therapeutic Targets in Pancreatic Adenocarcinoma
- ▶ Epigenetic Pharmacology
- ▶ Interventional Radiology for Pancreatic Cancer
- ▶ Metabolism in Pancreatic Cancer
- ▶ Palliative Management of Pancreatic Cancer
- ▶ Palliative Surgery in Advanced Pancreatic Cancer
- ▶ Precision Medicine Based on Next-Generation Sequencing and Master Controllers
- ▶ Role of Radiotherapy in Locally Advanced Pancreatic Cancer
- ▶ Therapeutic Endoscopy in the Management of Pancreatic Cancer
- ▶ Treatment of Recurrent Pancreatic Cancer After Surgery
- ▶ Vaccine Therapy and Immunotherapy for Pancreatic Cancer

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Surgical Resection for Pancreatic Cancer Using the International Study Group of Pancreatic Surgery (ISGPS) Classifications

Thilo Hackert, Christoph W. Michalski, and Markus W. Büchler

Contents

Abstract

The International Study Group of Pancreatic Surgery (ISGPS) has published a number of definitions within the last decade to standardize terminology and reporting in the field of pancreatic surgery. Furthermore, the group has also extended their approach of summarizing expert opinions in terms of recommendations for the surgical treatment of pancreatic cancer. These definitions and consensus statements have been highly accepted in the worldwide surgical community, and the citations of the respective papers are steadily increasing, which underlines their importance not only in clinical practice but also in the setting of study conductance and scientific reporting. Besides the initial definitions of postoperative complications (postoperative pancreatic fistula, hemorrhage, and delayed gastric emptying), the recent ISGPS publications have addressed important issues of

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pancreatic cancer (PDAC) surgery, especially with regard to preoperative evaluation of resectability, extended resections, and lymph node management during PDAC resection. Currently, more ISGPS publications are being prepared to cover the entire field of surgical and perioperative management in pancreatic surgery.

This chapter gives a general overview of the ISGPS definitions and consensus recommendations and, in addition, puts a special focus on the publications of the group dealing with PDAC surgery.

Keywords

Pancreatic cancer · International Study Group for Pancreatic Surgery · Consensus statement · Lymphadenectomy · Borderline resectable pancreatic cancer · Extended resection

Introduction

The International Study Group of Pancreatic Surgery (ISGPS) has originally been introduced in 2005 as the "International Study Group of Pancreatic Fistula (ISGPF)" which was formed as an expert panel including 37 pancreatic surgeons from all over the world with the initial aim to address the topic of the definition of postoperative pancreatic fistula (POPF) [1]. The background of this approach was the fact that in 2004, a total number of 26 different definitions for POPF were used in the international literature [2]. This caused a significant bias in reporting outcomes after pancreatic resections with the consequence that POPF rates were neither comparable nor was a valid examination of outcomes between studies possible. In the light of this problem, the ISGPF introduced a standardized POPF definition with a grading system to reflect POPF severity [1]. Fulfilling the criteria of an easy clinical application, this initial definition was therefore quickly accepted by most centers and became the standard reporting tool for POPF in retrospective as well as prospective studies. With an average number of 150 citations per year, this publication has been cited more than 1,800 times (July 2016) which gives an impression of its relevance in the field of pancreatic surgery [3]. Currently, the ISGPS has decided to update the POPF definition as in the meantime, it has been shown that there are some points of debate, especially with regard to percutaneous drainage in the management of these patients [4]. A revised classification will be proposed in autumn 2016. Apart from POPF, the ISGPS has established definitions on postpancreatectomy hemorrhage (PPH) [5] and delayed gastric emptying (DGE) [6]. Both of these definitions are applied by most authors in the meantime and have also led to a more standardized reporting on morbidity of pancreatic resections. Furthermore, reporting systems and consensus statements on postoperative chyle leaks and pancreatic anastomoses are currently in preparation [7].

With specific regard to pancreatic cancer (PDAC) surgery, three definitions and consensus publications by the ISGPS were recently published in 2014,

aiming at standardized procedures and reporting. They cover the topics of lymphadenectomy [8], extended procedures [9], and borderline resectability [10] including the controversial field of neoadjuvant treatment versus upfront resection in the respective patients. During preparation of these three recommendations, the current evidence has been systematically collected, reviewed, and condensed under consideration of other already existing guidelines (i.e., the National Comprehensive Cancer Network (NCCN) guidelines). Finally, the expert panel of pancreatic surgeons has voted on the statements of every topic and has approved the recommendations which are consequently given with a comment on the strength of evidence and agreement.

Based on the ISGPS recommendation for staging of PDAC [10], a contrast-enhanced computed tomography (CE-CT) using a pancreas-specific protocol should be the gold standard to determine local tumor extension, exclude liver metastases, and evaluate a possible vascular infiltration. The CE-CT should offer a visualization and differentiation of normal and tumorous pancreatic tissue in an arterial and venous phase including an optimal contrast imaging of the vascular structure in both phases as well as a visualization of the liver parenchyma. For the definition of local resectability in PDAC, the extension of the tumor toward the superior mesenteric vein (SMV)/portal vein (PV) and the celiac axis (CA) as well as the superior mesenteric artery (SMA) is of utmost importance. CE-CT is available in nearly all institutions and offers sensitivity and specificity rates of 63–82% and 92–100%, respectively, with regard to PDAC diagnosis. The use of a pancreas-specific CE-CT examination protocol with a 30° right-sided position of the patient and oral water intake to enhance the contrast in the gastroduodenal region is the basis to maximize accuracy in the preoperative diagnostics [11]. In case of contraindications for a CE-CT, magnetic resonance imaging (MRI) can be used instead of CE-CT as the accuracy of MRI is comparable to CE-CT regarding diagnosis of PDAC and evaluation of the local tumor extension [12].

The ISGPS criteria for local resectability [10] are mainly based on the recommendations of the National Comprehensive Cancer Network [13]. Resectability is defined as primary resectable PDAC, borderline resectable PDAC (BR-PDAC), or unresectable PDAC. The terms "unresectable," "irresectable," and "locally advanced" PDAC are mostly used as synonyms indicating that no upfront resection is possible but that the tumor is still locally limited and no distant spread is present.

Resectable PDAC is characterized by the absence of any vascular attachment (no distortion of SMV or PV) and clearly preserved fat planes toward CA and AMS. BR-PDACincludes findings with a distortion/narrowing or occlusion of the respective veins but a technical possibility of reconstruction on the proximal and distal margin of the veins (Fig. 1). With regard to the arterial structures, a semi-circumferential abutment ($\leq$180°) of the SMA or an attachment at the hepatic artery (HA) without contact toward the CA is also regarded as BR-PDAC. Unresectable PDAC is defined as a more extended involvement of the SMA, CA, aorta, or inferior

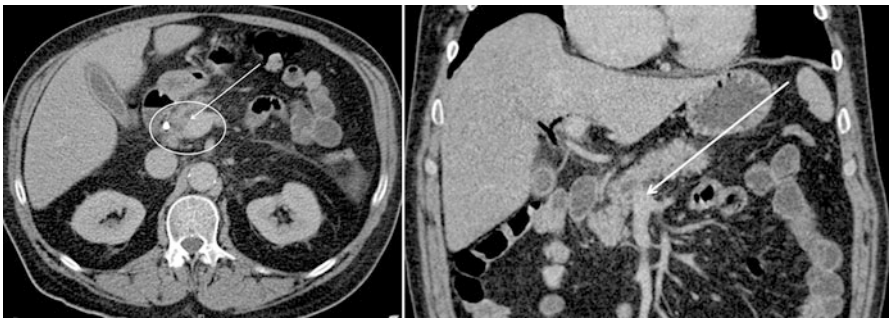

Fig. 1 BR-PDAC of the pancreatic neck. Contrast-enhanced CT scan (porto-venous phase, axial left, and coronary reformatting right side). Tumor (*white circle*) with contact to the porto-venous confluence (*white arrow*)

vena cava as well as a SMV/PV venous involvement without a possibility for surgical reconstruction of the venous tract due to the lack of a suitable luminal diameter of the feeding and/or draining vein. This situation is most likely to be found in tumor-associated portal cavernous transformation.

For the consecutive therapeutic decision, the recommendations for resectable and irresectable PDAC are clearly defined. While patients with resectable PDAC should undergo surgical exploration and radical resection, for unresectable PDAC patients, the option of neoadjuvant treatment should be considered as the therapy of choice with the chance of a reevaluation and eventually surgical exploration (see below). In BR-PDAC, therapeutic decisions have to differentiate between venous and arterial vessel involvement. Consequently, some authors differentiate between these two situations and define venous BR-PDAC (BR-PV) and arterial BR-PDAC (BR-A) as separate findings, although this subclassification is not included in the original ISGPS publication. In venous BR-PDAC, upfront surgery should be performed and – if the intraoperative finding matches the presumed borderline situation as defined above – completed as an en bloc tumor removal with venous replacement [14]. In contrast, when suspected arterial BR-PDAC is intraoperatively found to be a true arterial involvement, no general recommendation for resection is given; neoadjuvant treatment with a consecutive reexploration and the option for a secondary resection is possible as well as direct arterial resection in exceptional cases or under study conditions.

Exceeding the topic of vascular tumor involvement, the involvement of any adjacent organ, i.e., the mesocolon, colon, stomach, adrenal gland, or kidney, may be regarded as BR-PDAC as well. Although this is not covered by the ISGPS definition for BR-PDAC, surgery for respective findings is defined as an extended approach by the ISGPS [9]. There is international consensus that these extended approaches are feasible in terms of surgical and oncological outcome, and organ involvement should not be considered an obstacle for resection as long as a radical

tumor removal is possible. Consequently, these patients should undergo upfront surgery and should not be treated in a neoadjuvant setting [9].

ISGPS Definitions on Surgical Procedures and Resectability

Lymphadenectomy During PDAC Surgery

The extent of lymphadenectomy has been under debate since the late 1990s and numerous studies have been conducted on this question [15–21]. To define lymph node positions and classify intra- and postoperative findings, the lymph node classification of the Japanese Pancreatic Society [22] has been accepted worldwide and is used in most scientific publications. Consequently, this nomenclature has also been chosen as the basis of the ISGPS consensus statement [8]. According to this system, the peripancreatic lymph nodes can be divided into three groups (1st, 2nd, 3rd order) of regional lymph nodes that are further subdivided in some positions such as the hepatoduodenal ligament (group 12a, b, c, h, p). This classification is helpful not only clinically to describe lymph node spread in detail but also to make studies on lymph node dissection comparable (Fig. 3).

Another important topic in the ISGPS statement is the number of lymph nodes that should be retrieved as a minimum prerequisite for a valid pathological staging. As a low number of harvested lymph nodes bears the risk of understaging and a consequent N0 classification may not be an actual N0 stage as positive nodes may have been left in situ, the minimum number of examined lymph nodes during any type of PDAC surgery should be 15 nodes [8]. After neoadjuvant treatment, a lower number of lymph nodes were defined to be acceptable as in this situation; less lymph nodes may be identified even with a most accurate pathological examination of the specimen. Furthermore, in all cases, the lymph node ratio should be provided in the pathological report according to the ISGPS recommendation as a lymph node ratio of >0.2 has been shown to be a negative predictor of survival [23–25].

Partial duodenopancreatectomy includes a standardized lymphadenectomy, which contains the lymph nodes of the hepatoduodenal ligament (group 12b and c), along the common hepatic artery (group 8a), the cranial portion of superior mesenteric vein (group 5 and 6), as well as right-sided lymph nodes of the superior mesenteric artery (group 14a and b) and the peripancreatic nodes (group 13 and 17, Fig. 2) [8]. The impact of extended lymph node dissection (i.e., in the interaortocaval space, left side of the celiac trunk and superior mesenteric artery) has been investigated in several randomized controlled trials between 1998 and 2012 [15–20]. Although there were certain differences in the studies with regard to the number of resected lymph nodes (20 vs. up to 40), most of the authors could not show any survival difference in the study collectives, neither in N0 nor in N1 patients that underwent standard or extended resections. Only Pedrazzoli et al. [15] found a

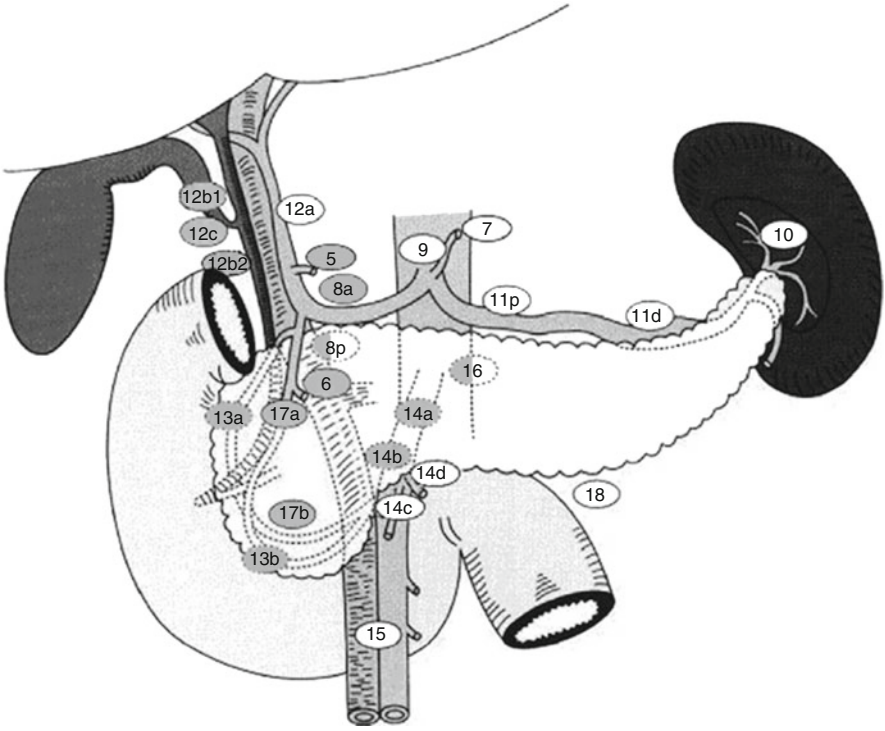

Fig. 2 Standardized lymphadenectomy during pancreatoduodenectomy according to the ISGPS consensus (Adopted from Ref. [8]). Dissected lymph node stations are marked in *gray*

survival benefit of 7 months in the subgroup analysis for N1 patients that underwent extended resection. Furthermore, all authors besides Pedrazzoli et al. observed a significantly increased surgical morbidity or decreased quality of life in the postoperative follow-up.

Two meta-analyses published in 2007 and 2009, respectively [21, 26], analyzed these studies – with regard to their individual scientific quality and results. No benefit for an extended approach of lymph node dissection could be concluded with respect to tumor control and survival. Furthermore, an increased rate of perioperative complications and a decreased quality of life were demonstrated. Therefore, with regard to these studies and consequently based on a level 1 evidence, the concept of extended lymphadenectomy is not recommended in PD as stated in the ISGPS consensus.

Considering distal pancreatectomy (DP), lymph node involvement is mainly observed in the peripancreatic lymph nodes along the body and the tail of the gland [27]. Further frequent metastases sites are the nodes along the splenic artery, the para-aortic area, and along the inferior margin of the pancreas as well as along the superior mesenteric artery. The regional lymph nodes attached to the pancreas, along the inferior margin (group 18) and at the splenic artery (group 11), are routinely

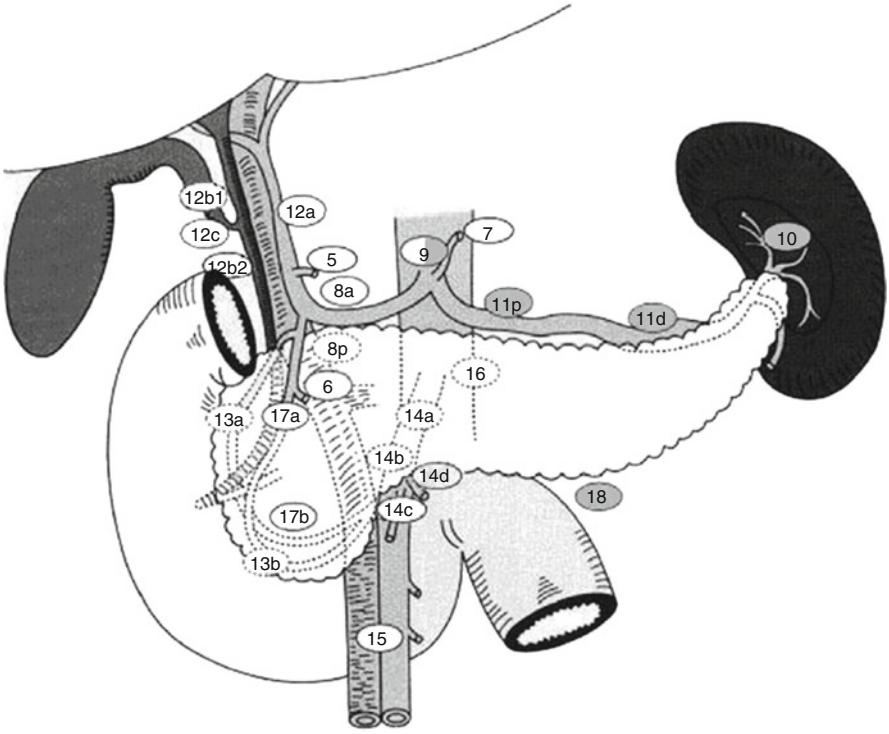

Fig. 3 Standardized lymphadenectomy during distal pancreatectomy according to the ISGPS consensus (Adopted from Ref. [8]). Dissected lymph node stations are marked in *gray*

removed during DP as well as group 10 in the splenic hilum as a standard splenectomy should always be performed in DP for PDAC [28]. The lymph nodes at the basis of the celiac axis (group 9) should be resected according to the ISGPS statement in case of tumors of the pancreatic body (Fig. 3). Para-aortic dissection (lymph node groups 7 and 16) is not recommended as a standard procedure [8].

Lymphadenectomy in total pancreatectomy, which is usually performed en bloc with splenectomy for PDAC, is not explicitly defined by the ISGPS. However, it seems reasonable to regard total pancreatectomy as a combination of PD and DP and combine standard lymph node dissection of both procedures. This procedure will usually result in approximately 30–50 lymph nodes included in the resected specimen. Interaortocaval lymph node resection during total pancreatectomy can be regarded as an extended approach and should not be performed as a routine procedure.

However, the ISGPS recommendation explicitly states that no definitive consensus is currently reached regarding the prognostic impact of para-aortic lymph nodes on one hand and that – in addition – there is no recommendation on how to proceed in cases where lymph nodes outside the standard resection area are found to be positive intraoperatively [8]. The issue of continuing or terminating resection in

this case remains unsolved and is handled differently in centers around the world to date.

Borderline Resectable PDAC Including Vascular and Extended Resections

Venous Resections

Vascular resections during pancreatoduodenectomy to achieve tumor clearance and improved survival in case of SMV/PV involvement have been advocated for the last three decades [29]. Today, these approaches have gained wide acceptance and been included in national and international guidelines around the world [10, 13], although most recent publications question this approach with regard to morbidity and oncological outcome [30]. However, they do not conclude that venous resections should generally not be performed but that a greater emphasis should be put on preoperative patient selection. According to the ISGPS, venous involvement is neither a criterion for unresectability nor for neoadjuvant therapy as long as the technical possibility to restore vessel continuity is given [10]. This must be intraoperatively confirmed by evaluation of the diameter of the proximal and distal vein before resection. There are mainly four types of PV/SMV resection defined by the ISGPS [10]. In case of minimal tumor adherence to PV/SMV, resection can be performed as a tangential resection of the vein with a direct suture (type 1 reconstruction). This is possible for pancreatoduodenectomy as well as for distal pancreatectomy if the direct suture closure does not lead to a hemodynamically relevant stenosis and a consecutive risk of acute postoperative thrombosis. The second possibility is the closure of a short tangential defect by a patch insertion (type 2, Fig. 4). This patch can either be harvested from a homologous venous graft (i.e., saphenous or internal jugular vein) or – as a suitable alternative – a peritoneal patch can be harvested and used with the peritoneal surface directed toward the lumen of the vein [31]. If this is not possible due to the length of the resected segment, the mesenteric root can be mobilized completely by resolving the

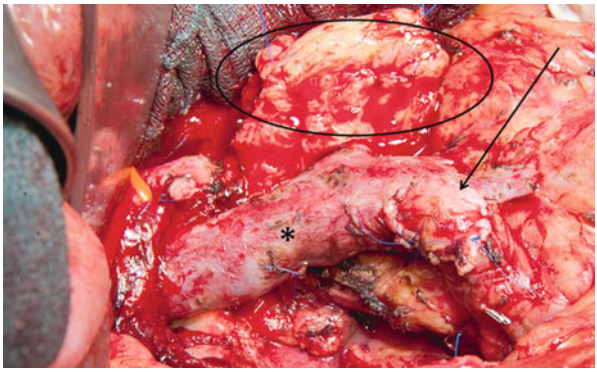

Fig. 4 Intraoperative view after ISGPS type 2 (peritoneal patch) reconstruction of the superior mesenteric vein. Patch (*black arrow*) on the right lateral superior mesenteric vein, portal vein (*black star*), and cut margin of the pancreas (*black circle*)

attachment of the right hemicolon to the retroperitoneal adhesions (Cattell-Braasch maneuver [32]) and a direct anastomosis in an end-to-end fashion. If this is impossible, the interposition of a vascular graft, using either autologous structures (saphenous vein, left renal vein, or internal jugular vein) or allogenous synthetic grafts (ringed polytetrafluoroethylene (PTFE) graft), can be performed (type 4 reconstruction) [33].

The feasibility of SMV/PV resection has been demonstrated in large series that showed surgical morbidity and mortality rates comparable to pancreatic head resections without vascular involvement [34, 35] as well as in a recent systematic review including data on the outcome of more than 1,600 patients from 52 publications [14]. With a median operation time of 8.5 h and a median blood loss of 1,750 ml, SMV/PV resections resulted in an average perioperative mortality of 5.9% and overall morbidity of 42%.

Even if preoperative diagnostics show a tumor-related complete obstruction of the portal vein, this must not be regarded as a contraindication for surgery. Although intraoperatively, the preparation may be more difficult due to the collateral vessels, the restoration of the portal venous flow after resection and anastomosis offers an adequate drainage of the bowel despite the removal of most of the collateral vessels that may be necessary during the preparation.

Oncological outcome in patients with venous resections is similar to patients undergoing standard resections for PDAC without increased rates of local or systemic failure [14]. The rate of histologically proven SMV/PV invasion is app. 65%, and in addition, a positive lymph node stage is found in two thirds of the patients, too. These findings result in a 1-, 3-, and 5- year overall survival of app. 50%, 18%, and 8% [14, 34] which is clearly superior to any type of palliative treatment. To address the high percentage of positive nodal findings in patients who undergo portal or superior mesenteric vein resection, the importance of adjuvant therapy has to be underlined. Since adjuvant treatment has strongly improved survival and been introduced as the standard of care for patients with pancreatic cancer, future survival rates of patients with venous resection should be even better than those reported so far. SMV/PV resections during PDAC surgery can therefore be regarded as a standard procedure to achieve a complete removal of the tumor and can also be performed during multivisceral resections with the same intent of complete tumor clearance [36].

Arterial Resections

In contrast to venous tumor adhesion, arterial encasement of CA or SMA can be regarded as a symptom of a very aggressive tumor biology, and the decision to perform a surgical resection in this situation is highly individual and still regarded as an extraordinary approach in PDAC surgery [37, 38]. According to the ISGPS, the extent of arterial encasement determines the classification of the finding as BR-PDAC or unresectable PDAC as described before.

Both major arterial structures – CA and SMA – have to be evaluated differently with regard to the performance of a pancreaticoduodenectomy or a distal pancreatectomy and the extent of tumor abutment. If the SMA is involved in the tumor

process exceeding 180° of the circumference or in case of CA abutment, this is rather a general exclusion criterion for resection, and tumor resection with arterial reconstruction has only been reported in few patients [37]. In contrast, situations with an arterial tumor abutment <180° along the SMA or short-segment abutment of the HA as the only vitally important structure of the CA must not be considered as irresectable but fulfil the criteria of BR-PDAC [10].

There is consensus that all patients with suspected BR-PDAC due to an arterial involvement should undergo surgical exploration to confirm this situation and decide on the consecutive therapy. To evaluate arterial infiltration along the SMA and/or CA, "artery-first" approaches can be useful [39, 40]. These techniques describe the preparation of the SMA or CA as an initial step before reaching any "point-of-no-return" situation during surgery. In the meantime, more than six different techniques have been described as "artery-first" techniques and are used according to the respective surgeons' or centers' preference [41].

In case of confirmed tumor infiltration of the CA or the SMA, palliative treatment is recommended as the standard of care. However, the possibility of arterial resections as an individual approach or within clinical trials and the consideration of a neoadjuvant treatment with a consecutive reexploration have to be mentioned. On one hand these approaches have been reported especially during distal pancreatectomy; on the other hand the topic of neoadjuvant therapy of BR-PDAC is currently one of the most important fields in PDAC treatment.

Regarding distal pancreatectomy, CA resection without revascularization (modified Appleby procedure) is an option for tumor removal as long as the proper hepatic artery is preserved and a sufficient arterial inflow via the gastroduodenal artery is present. Including approximately 200 patients, numerous case series have described this procedure with reasonable results in terms of surgical and oncological outcome which seems to be nearly equal to the standard approaches [42–44]. According to the larger series in the literature, that include more than ten patients, these procedures can be carried out with mortality rates of 0–7% and an average overall morbidity of app. 50%. Median survival in these reports ranges between 10 and 25 months; in the majority of publications, app. 20 months can be achieved. According to these retrospective studies, CA resection during distal pancreatectomy seems to be a considerable option in terms of postoperative and long-term outcome; however, no high-level evidence is available to support these findings.

In case of resection of the HA or SMA during pancreatoduodenectomy or total pancreatectomy, restoration of the arterial perfusion has to be performed either with a direct anastomosis or graft insertion to replace the resected vessel. This reconstruction can be done with an interposition of any arterial vessel of the celiac axis or a venous interposition graft. In a recent review, the role of arterial resection has been critically evaluated including all currently available studies [38]. Regarding resection of the SMA, five studies were identified, including a total number of less than 30 patients. All authors showed that the resection is technically possible; grafting with the saphenous vein was the most commonly used method for reconstruction. However, morbidity of this approach is high and the oncological outcome is not yet

convincing from the limited evidence. Overall, CA or HA resection is performed more often than SMA resection. Surgical morbidity is up to 40%, and mortality in pancreaticoduodenectomy with arterial resection ranges from 0 to 35%, showing the inconsistent data basis of this approach. The major risk following HA reconstruction is the occurrence of arterial hepatic perfusion failure that may cause acute problems postoperatively in terms of liver ischemia, necrosis, and infection with a high associated mortality [45, 46]. Besides the operative complications in procedures with arterial resections, even more importantly, the mentioned meta-analysis showed a poor oncological outcome with significantly impaired survival in comparison to standard PDAC resections [38]. Consequently, resection of arterial vessels during PDAC surgery does not represent a standard procedure. It may be a feasible option with regard to distal pancreatectomy and en bloc CA resection under preservation of the proper HA without reconstruction of a major arterial vessel. All other arterial resections are highly individual approaches for selected patients and need to be carried out by experienced pancreatic surgeons to minimize postoperative complications.

Multivisceral Resections

Beyond infiltration of vascular structures, also adjacent organs can be affected by locally advanced PDAC. Mainly, the colon, stomach, left adrenal gland, small bowel, and left kidney are affected. A complete tumor removal therefore requires partial or total resection of these organs during partial, distal, or total pancreatectomy. These multivisceral resections fulfil the criteria of "extended resections" defined by the ISGPS in 2014 [9]. A neoadjuvant treatment is not indicated, if a complete resection seems to be technically possible on the basis of the preoperative cross-sectional imaging. In larger series reporting on multivisceral resections, 20 up to more than 270 patients are included [36, 47]. The most commonly resected organs are the colon and stomach in case of partial or total pancreatectomy and the adrenal gland during distal pancreatectomy. Remarkably, also PV/SMV resections are often performed synchronously reflecting the local extension of the tumor and the close anatomic relationship of these venous structures.

The currently largest single-center series from Heidelberg included 101 patients and showed that multivisceral resections were associated with an increased postoperative morbidity but not mortality [36]. Postoperative morbidity was predicted by a long operation time and a resection of two or more additional organs as independent risk factors for intra-abdominal complications or need for relaparotomy. Regarding oncological outcome, survival was similar to standard resections. In a study on 55 patients with multivisceral resections for PDAC [48], median survival was 16 months versus 18 months for standard resections, which was significantly better than palliative bypass surgery. Multivariate risk factors for postoperative morbidity during multivisceral resections in this study included intraoperative blood transfusion and nephrectomy, whereas survival was determined by T status, kidney resection, resection of four or more organs, any postoperative transfusion, and intensive care unit stay of >2 days in the univariate analysis, and T status alone was confirmed as a predictor of survival in the multivariate analysis.

A present update of the first study analyzing 600 PDAC patients who underwent extended resections for BR-PDAC compared to 1,200 standard resections confirms the mentioned findings [49]. The performance of extended resections is associated with increased postoperative morbidity and mortality for patients with relevant comorbidities and operation times of more than 5 h. Apart from these two risk factors, multivisceral and vascular resections were not identified as parameters for poor postoperative outcomes. Extended PDAC resections resulted in 16 months median and 11% 5-year survival, which is clearly superior to any palliative treatment option. On one hand, these results underline that extended surgery is a feasible approach; on the other hand, they raise the unsolved question of an accurate patient selection as certain subgroups seem to have a much greater benefit from surgery than others, and valid preoperative markers for this stratification are not defined yet.

Neoadjuvant Treatment for BR-PDAC and Unresectable PDAC

Today, there is no sufficient evidence to support neoadjuvant treatment in resectable PDAC which is clearly stated in the ISGPS consensus [10]. Neither chemoradiation nor chemotherapy alone has shown a benefit in this situation [50]. Comparably, in case of BR-PDAC, no neoadjuvant treatment is recommended for venous tumor adherence or involvement of adjacent organs if a resection is technically possible and complete tumor removal can be achieved. According to the consensus recommendation of the ISGPS, these patients should undergo upfront resection as well [10]. In this context, it must be emphasized that there are a number of ongoing studies on this issue that evaluate the effect of neoadjuvant therapy in the abovementioned situations, and more evidence-based result are expected in the next 5–10 years [51–56]. The possible advantages of neoadjuvant therapy could include a stratification of patients with regard to tumor biology indicating those subgroups of patients with a very aggressive tumor that would not benefit from a resection despite the absence of systemic spread at the time of diagnosis. In addition, neoadjuvant treatment could improve R0 resection rates and decrease the incidence of local recurrence. Therefore, the results of these studies are highly warranted and may change clinical practice, comparable to studies investigating esophageal-gastric cancer outcome during the early 2000s and establishing the recommendation and international agreement on neoadjuvant treatment for the majority of these patients, today [57].

In case of BR-PDAC or clearly unresectable PDAC due to arterial involvement, neoadjuvant treatment should be considered instead of upfront surgery [10]. This consideration is based on the fact that arterial resections – although often technically possible – are associated with a significant increase in postoperative morbidity as well as mortality. Moreover, even patients after successfully undergone arterial resection often have a very limited oncological benefit and suffer from early recurrence or metastatic spread [38]. These limitations can be overcome by neoadjuvant therapy as on one hand a patient selection can be conducted, because patients showing a tumor progression will be excluded from surgery. On the other hand, an arterial resection can be avoided during surgery in a considerable number of patients. If only fibrosis is found along the arterial structures instead of former vital tumor

formations, dissection of the arteries instead of resection can be performed. This clinical observation raises the question of diagnostic accuracy of the restaging after completion of neoadjuvant treatment. Many patients do not show an explicit downstaging of the local findings in CT scans after chemotherapy or chemoradiation [58].

As perineural spread has been shown to be an important prognostic factor [59], this has been investigated in several studies with regard to preoperative imaging prediction [60, 61]. Although in primary diagnosis, high-resolution CT scan can predict perineural invasion along larger vessels with an accuracy of 95% [61], this does not seem to be reliable in a post-neoadjuvant setting and diagnostic sensitivity and specificity are highly limited [58].

An additional particular challenge of restaging is as the differentiation of vital tumor and fibrosis by conventional cross-sectional imaging is limited and even PET-CT scans do not offer 100% accuracy [62]. Patients with a clear tumor progression under neoadjuvant treatment should be excluded from secondary exploration. As to date, no valid diagnostic modality or marker is available that differentiates between vital tumor and residual fibrotic tissue with a sufficient sensitivity and specificity; all other patients should undergo surgical exploration to definitely evaluate this and perform a resection whenever possible. Intraoperatively, after confirming the absence of vital tumor by frozen section, a resection is often possible and eventually an ypT0 situation may be found. Due to the three scenarios described, neoadjuvant treatment is helpful to stratify patients and recognize those with BR-PDAC, who do not benefit from extended resections.

The debate on the most effective neoadjuvant treatment scheme is a currently unsolved issue. Traditionally, chemoradiation for locally advanced PDAC using gemcitabine- or 5FU-based protocols along with 50–54 Gy of radiation has been used [63] and shown secondary resection rates of app. 30% [64]. With the introduction of highly effective chemotherapy regimens such as Folfirinox (5-FU, leucovorin, irinotecan, oxaliplatin) or nab-paclitaxel, this approach has been challenged [65–67]. Based on the observations in metastatic disease, where this therapy has been shown to be significantly more effective than gemcitabine [68], several studies have reported on its efficacy in the neoadjuvant setting. For borderline resectable and locally advanced findings, a secondary resection rate of 85% was found in a 40-patient study; however, 24 of the included patients received an additional chemoradiation before surgery [58]. For locally advanced disease, a recent study on 575 patients receiving different chemotherapy regimens found that the highest secondary resection rate (61%) was observed for the subcollective of 125 Folfirinox patients [69]. As there are no randomized studies comparing these approaches, evidence-based recommendations on the best treatment option cannot be given, but a Folfirinox-based regimen seems to be the most promising approach.

To facilitate patient selection with BR-PDAC for the most promising therapy (upfront resection vs. neoadjuvant treatment), various prognostic scores and parameters have been examined. Imaging criteria (i.e., suspicion of lymph node metastases) and clinical performance status were used in a publication by Katz et al. [70] but did not reliably predict prognosis. Currently, the modified Glasgow Prognostic Score (mGPS) and CA 19–9 levels are the most reliable prognostic parameters [71,

72]. Especially a decrease or even normalization of elevated CA 19–9 during neoadjuvant treatment is associated with a good prognosis [72, 73]. The mGPS – although not as commonly used as CA 19–9 – seems to be an additional valid predictor as a score value of two can be regarded as a poor prognostic outcome parameter in the neoadjuvant setting [74]. Other biomarkers or genetic specifications cannot yet be recommended for prognostic stratification or therapy decisions [10].

Conclusion

The International Study Group of Pancreatic Surgery (ISGPS) has standardized preoperative classifications and postoperative outcome definitions in pancreatic surgery. These definitions are accepted as a standard in the pancreatic surgery community and are valued because they – for the first time – allow for outcome comparisons across different institution. These definitions are updated regularly by the study group members and include statements on postoperative complications, preoperative assessment of resectability, extended resections, and lymph node management.

Cross-References

- ▶ Arterial Resection in Pancreatic Cancer
- ▶ Borderline Resectable Pancreatic Cancer
- ▶ Clinical Decision-Making in Pancreatic Cancer
- ▶ Interventional Radiology for Pancreatic Cancer
- ▶ Neoadjuvant Chemotherapy in Pancreatic Cancer
- ▶ Staging and Postoperative Outcomes Using the International Study Group of Pancreatic Surgery (ISGPS) Classifications

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Venous Resection in Pancreatic Cancer Surgery

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Abstract

Because of their anatomical proximity to the pancreatic head, the portal vein (PV) and superior mesenteric vein (SMV) are frequently involved in pancreatic head cancers. PV/SMV resection and reconstruction should be arranged according to the degree of PV/SMV invasion. In case of minimal invasion, the PV/SMV wall can be partially resected and repaired with direct suture, or a patch repair using a vein graft can be performed. In case of wide invasion of the lateral aspect of the vein or circumferential involvement, segmental resection of the PV/SMV should be performed. Reconstruction in this case will be performed using either direct end-to-end anastomosis or the interposition of vein graft. When the confluence of the splenic vein (SV) is involved and ligation and division of the SV is performed, varices caused by left-sided portal hypertension may form in the late phase after surgery. In such cases, preservation of the omentum and the transverse and right colic marginal vein is important to maintain the collateral route and to avoid the formation of varices. Several reports have indicated that pancreatectomy with PV/SMV resection can be performed with acceptable morbidity and mortality. Moreover, survival is comparable for patients with and without PV/SMV resection, although some reports indicate that the prognosis in patients with PV/SMV resection is worse than that of patients who do not undergo PV/SMV resection. The clinical benefit of PV/SMV resection for pancreatic cancer is still controversial.

Keywords

Regional pancreatectomy · Extended lymphadenectomy · Vein graft interposition · Autologous vein graft · Homologous vein graft · Left-sided portal hypertension · Portal vein thrombus · Portal vein stent graft

Introduction

Pancreatic head cancer spreads quickly to the adjacent tissues and distant organs. In addition to the regional lymph nodes, pancreatic cancer invades the retropancreatic neural tissue, duodenum, portal vein (PV), superior mesenteric vein (SMV), and superior mesenteric artery (SMA). For this reason, aggressive surgery that completely removes the cancerous lesion and surrounding tissues was recommended in the early 1970s. Fortner proposed an extensive surgical procedure called "regional pancreatectomy," which permits en bloc resection of the pancreatic segment of the PV/SMV, the celiac axis, and the proximal portion of the SMA together with the lymph nodes and lymphatic vessels. However, this aggressive procedure unexpectedly failed to improve patients' survival and resulted in unacceptable short-term surgical outcomes with high morbidity and mortality [1, 2]. Nevertheless, the "regional pancreatectomy" proposed by Fortner encouraged Japanese surgeons in high-volume centers, and they performed radical surgery for pancreatic cancer in the

1980s [3, 4]. Their reports showed a benefit of radical surgery for advanced pancreatic cancer in terms of overall survival. One of the major flaws of these reports, however, was that they were all retrospective case control analyses and did not include any randomized controlled trials (RCT). Therefore, the benefit of performing radical pancreatectomy in patients with pancreatic cancer was still controversial.

In the 1980s, an Italian group performed the first RCT that compared standard pancreatoduodenectomy (resection of only peripancreatic tissue) and extended pancreatoduodenectomy (thorough resection of neural and lymphatic tissues, including the lymph nodes in the hepatoduodenal ligament and the nerve plexus around the SMA) for pancreatic head cancer [5]. Subsequently, similar RCTs (although there were minor differences in the protocols) have been performed in the United States [6–8], Japan [9], and Korea [10] from the 1980s to the 2010s (Table 1). Interestingly, all of the RCTs failed to show any survival benefit for extended pancreatoduodenectomy despite its high postoperative morbidity rate and negative impact on short- and long-term quality of life. These results clearly indicated that the use of routine "prophylactic" extended lymphadenectomy for pancreatic head cancer may not be necessary to improve survival. However, they do not mean that radical surgery to achieve R0 resection is not worthwhile. Considerable evidence has shown a better prognosis in patients who underwent R0 resection compared with those who underwent R1 or R2 resection for pancreatic cancer [11, 12]. In fact, when the tumor is resected with R0 status irrespective of the extent of surgery, the median survival of patients may be better for those treated with best currently available combination chemotherapy such as FOLFIRINOX or gemcitabine and nab-paclitaxel [13, 14]. Moreover, recent reports have indicated that adjuvant chemotherapy substantially improved survival in patients who underwent resection for pancreatic cancer [15–17]. Therefore, surgeons must make every effort to achieve R0 resection for pancreatic cancer. Radical pancreatectomy that includes the extensive resection of peripancreatic tissues and organs should be permitted for this purpose.

The PV and SMV are the most commonly involved vessels when pancreatic cancer involves the pancreatic head. Even after five RCTs failed to find a survival benefit for "prophylactic extended surgery" for pancreatic head cancer, many surgeons continue to combine the resection of PV/SMV when the tumor has invaded this vessel and when they can achieve R0 resection by resecting this vessel. However, since there is no RCT focusing on the clinical value of PV/SMV resection, the benefit of resecting PV/SMV remains unclear. Nevertheless, it is important to elucidate the value of PV/SMV resection in pancreatic cancer through the accumulation and analysis of currently available best practice data. This chapter discusses the pattern of PV/SMV involvement in pancreatic cancer, the surgical techniques for PV/SMV resection and reconstruction, complications related to PV/SMV resection, and the clinical value of combined PV/SMV resection when performing pancreatectomy for pancreatic cancer.

Table 1 Comparison of 5 RCTs

	Italy		USA				Japan		Korea	
			Johns Hopkins[a]		Mayo Clinic					
Study period	1991–1994		1996–1997		1997–2003		2000–2003		2006–2009	
Published year	1998		1999 (first), 2002 (second)		2005		2012		2014	
	Standard	Extended	Standard	Extended	Standard	Extended	Standard	Extended	Standard	Extended
Number of patients	40	41	146 (80)	148 (82)	40	39	51	50	83	86
Operative time (min)	372	397	**354**	**384**	**378**	**450**	**426**	**547**	**356**	**420**
Blood transfusion (U)	1.95	2.07	0.5	0.5	**(22%)**	**(44%)**	2.1	2.4	0.1	0.25
PPPD/non-PPPD	20/20	23/18	125/21	148/0	0/40	0/39	19/32	23/27	62/21	60/26
PV resection	ND	ND	4 (3%)	4 (3%)	9 (23%)	8 (21%)	24 (47%)	24 (48%)	17 (21%)	23 (27%)
No. of lymph node retrieved	**13.3**	**19.8**	**17.0**	**28.5**	**15**	**36**	**13.3**	**40.1**	**17.3**	**33.7**
N (+), n (%)	24 (60%)	24 (59%)	(82%)	(77%)	(55%)	(68%)	32 (63%)	30 (60%)	57 (69%)	57 (66%)
R0, n (%)	29 (73%)	32 (78%)	**(80%)**	**(95%)**	(76%)	(82%)	48 (94%)	45 (90%)	71 (86%)	78 (91%)
Postoperative hospital stay (days)	22.7	19.3	**11.3**	**14.3**	13	16	43.8	42.4	19.7	22.8
Morbidity, n (%)	18 (45%)	14 (34%)	42 (29%)	64 (43%)	**Diarrhea 8%**	**Diarrhea 42%**	**Diarrhea 0%**	**Diarrhea 48%**	27 (33%)	37 (43%)
Mortality, n (%)	2 (5%)	2 (5%)	6 (4%)	3 (2%)	0	1 (3%)	0	1 (2%)	0	2 (2.3%)
Adjuvant treatment, n	IORT, 10	IORT, 9	CRT, 81	CRT, 83	CRT	CRT	None	None	CRT, 63	CRT, 59
Mortality, n (%)	2 (5%)	2 (5%)	6 (4%)	3 (2%)	0	1 (3%)	0	1 (2%)	0	2 (2.3%)
1-, 3-, 5-year survival (%)	ND	ND	75/34/13	73/38/29	82/41/16	71/25/17	78/28/16	54/18/6	45 (2 years)	36 (2 years)
Median survival (months)	11.2	16.7	30 (20)	28 (22)	26	18.8	19.9	13.8	18.8	16.5

Significant differences are found between the two groups in the date given in bold

ND not described, *PV* portal vein, *PPPD* pylorus-preserving pancreaticoduodenectomy, *non-PPPD* conventional pancreaticoduodenectomy or subtotal stomach-preserving pancreaticoduodenectomy, *IORT* intraoperative radiotherapy, *CRT* chemoradiotherapy

[a]Including patients with periampullary carcinoma and parentheses indicate the data for those with pancreatic cancer

Is Combined Vascular Resection Acceptable in Pancreatic Cancer Surgery?

Because of their anatomical proximity to the pancreatic head, the PV and SMV are frequently involved in pancreatic head cancers. To resect the tumor with negative surgical margins (R0 resection), wedge or segmental resection of the PV or SMV is necessary. The survival of patients who require PV/SMV resection can be worse than that of patients without PV/SMV invasion because of the high clinical stage and more active biological malignancy [18, 19]. However, previous reports, including two RCTs comparing pancreatic resection with PV/SMV resection and palliative treatment, showed that patients who underwent pancreatectomy with PV/SMV resection had better survival than those who underwent palliative surgery or radiochemotherapy [20–22]. Therefore, isolated PV/SMV involvement should not be a contraindication for pancreatic resection because postoperative morbidity and mortality rates following pancreatectomy with PV/SMV resection are acceptable [23–25]. When performing pancreatectomy with PV/SMV resection, it is important to completely remove the lesion that involves PV/SMV without violating the integrity of the tumor because a pathologically negative surgical margin is essential for improved survival [26].

Patterns of PV/SMV Invasion

There are several patterns of vascular invasion of pancreatic cancer to the PV and/or SMV. In 1992, Ishikawa et al. [27] proposed the angiographic typing of PV/SMV invasion using the portal phase of superior mesenteric artery (SMA) angiography. They classified angiographic findings into the following five types (Fig. 1): (1) normal, (2) smooth shift without narrowing, (3) unilateral narrowing, (4) bilateral narrowing, and (5) bilateral narrowing and the presence of collateral veins. According to these classifications, the postsurgical prognosis was very poor in patients with type IV or V, and their cumulative survival rates were almost identical to those of non-resectable patients. Conversely, a far better prognosis could be expected for type I and II patients. It should be noted that the portal phase of SMA angiography is likely to underestimate the true invasion of the PV/SMV. In the study by Ishikawa et al., angiography resulted in 40% underestimations, 54% correct diagnoses, and only 6% overestimations. The discrepancy may be explained by the fact that the internal diameter of the PV/SMV is unaffected when cancer invasion is limited to the tunica adventitia. Nakao et al. also presented a similar classification for PV/SMV invasion [28]. They classified PV invasion in pancreatic head cancer as type A (absent), B (unilateral narrowing), C (bilateral narrowing), or D (stenosis or obstruction with collaterals) (Fig. 1). Among a total of 358 patients with resected pancreatic head cancer, PV/SMV resection was performed in 21 out of 111 type A patients (19%), 77 out of 82 type B patients (94%), 96 out of 97 type C patients (99%), and all 68 of the type D patients (100%). No pathological PV/SMV wall invasion was observed in the 21 type A patients who underwent PV/SMV resection.

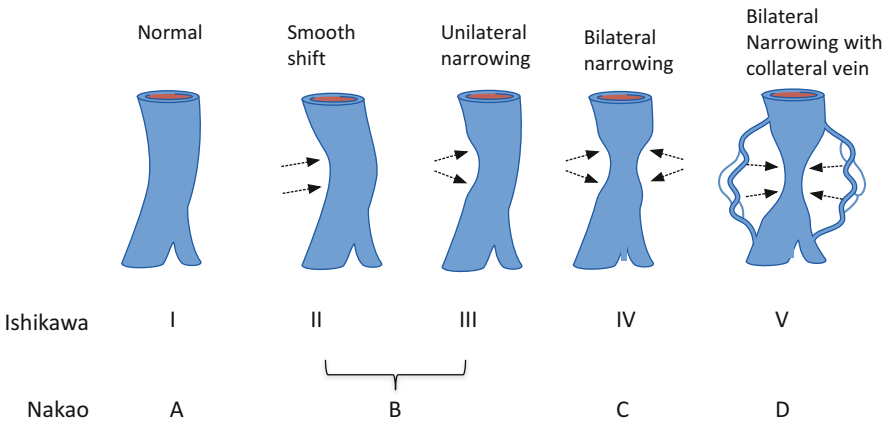

Fig. 1 Angiographic typing of PV/SMV invasion *Upper line*: Ref. [27]. *Lower line*: Ref. [28]

In contrast, the incidences of pathological PV/SMV wall invasion in types B, C, and D were 51%, 74%, and 93%, respectively. Moreover, the rate of tumor invasion into the tunica intima increased according to the radiographic type of PV/SMV invasion. In terms of prognosis after surgery, type A patients showed a significantly higher survival rates than other types. Type B patients had a significantly better prognosis than the type C and type D patients. No significant difference in survival rates was observed between patients with type C and D invasion, although those with type D had a higher survival rate than the unresectable group. Currently, the angiographic classification of PV/SMV invasion can be much less invasively determined using recent advances in multi-detector computed tomography (MD-CT) imaging [29–31].

Techniques for PV/SMV Resection and Reconstruction

Partial Resection and Reconstruction

In cases of minimal invasion of the lateral aspect of the vein, a vascular clamp is placed longitudinally, and the invaded part of the vein is resected and directly sutured. When PV/SMV stenosis is anticipated, the vascular clamps are placed proximally and distally, and transverse suturing after longitudinal resection should be applied to avoid stenosis of the reconstructed vein (Fig. 2a). The large defect can also be repaired with autologous vein grafts using the great saphenous vein, left renal vein, internal jugular vein, or synthetic material [23, 32–35] (Fig. 2b).

Segmental Resection and Reconstruction

In cases of wide invasion of the lateral aspect of the vein or circumferential involvement, vascular clamps are placed proximally and distally to the

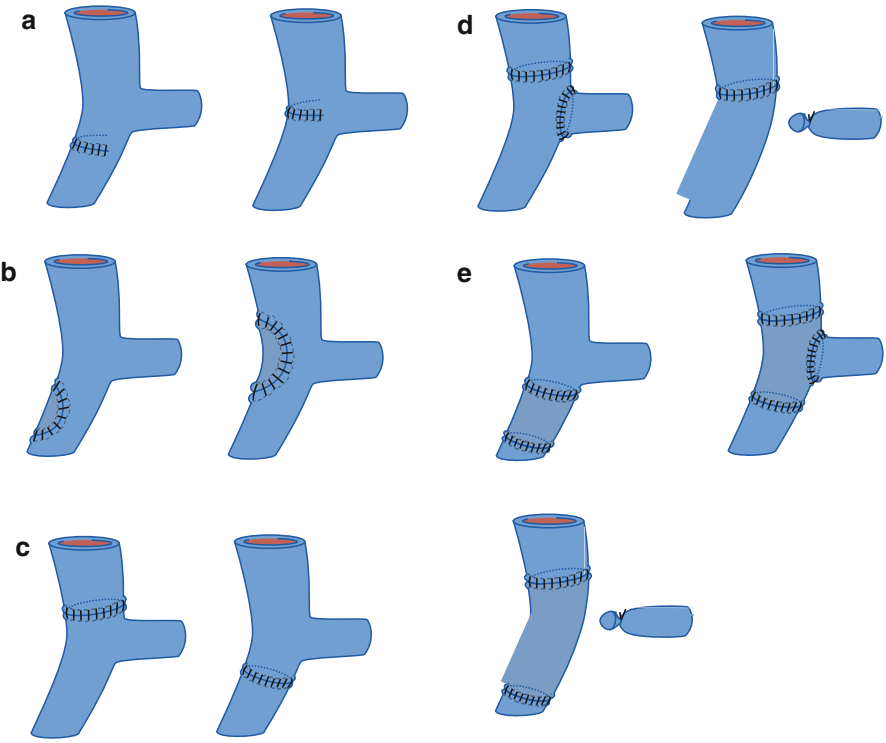

Fig. 2 Various patterns of PV/SMV resection and reconstruction

invaded portion, and segmental resection of PV/SMV should be performed (Fig. 2c). It is better to place the vascular clamps well apart from the invaded portion to retain sufficient flexibility for suturing the vessels (Fig. 3). When the PV/SMV defect is lengthy, full mobilization of the right hemicolon mesentery may help to approximate the proximal and distal resected ends of the PV/SMV.

There are two major procedures for the end-to-end anastomosis of PV/SMV or vein grafts. One is the "rotation method," and the other is the "intraluminal and over-and-over method." In case of the "rotation method" (Fig. 4), the vascular clamp is applied perpendicularly, and segmental resection of the involved vein is performed. First, the vascular clamps are turned 90° toward the right side, stay sutures are placed at the bilateral edges of the resected vein, and the left lateral wall is anastomosed using a continuous over-and-over suture technique. After the left lateral wall anastomosis is completed, the vascular clamps are turned back 180° toward the left side, and right-side wall anastomosis is performed using the continuous over-and-over suture technique. Next, the threads are tied after the proximal vascular clamp is removed, and the anastomosis is dilated. Finally, the distal vascular clamp is released. If the proximal cancer invasion has progressed near the jejunal veins or proximal or distal cancer invasion is found near the porto-mesenteric confluence, the previously described technique cannot be used before dissecting the SMA because

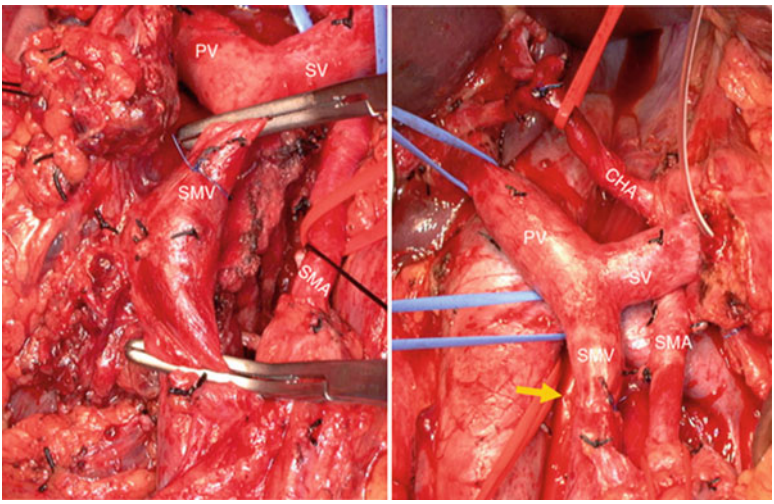

Fig. 3 Combined SMV resection and end-to-end anastomosis. *PV* portal vein, *SMV* superior mesenteric vein, *SV* splenic vein, *SMA* superior mesenteric artery, *CHA* common hepatic artery. An arrow indicates SMV anastomosis

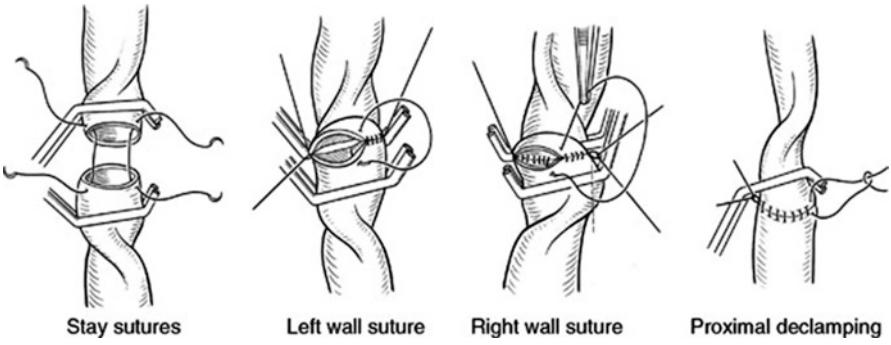

Fig. 4 Rotation technique for SMV/PV anastomosis

the PV/SMV cannot be turned 180°. In such instances, the "intraluminal and over-and-over method" is used (Fig. 5). In this method, the vascular clamps are placed horizontally near the jejunal vein and the splenic vein, and the involved vein is resected. For the reconstruction of the resected vein, stay sutures are placed at the bilateral edges of the resected vein, the intraluminal suture technique is applied for the posterior wall anastomosis, and the over-and-over suture technique is used for the anterior wall anastomosis.

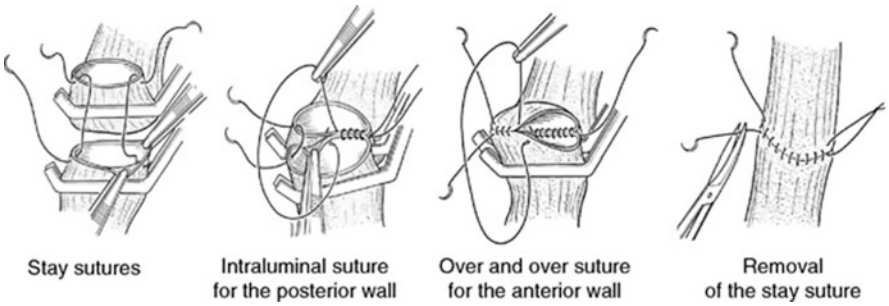

Fig. 5 Intraluminal and over-and-over techniques for SMV/PV anastomosis

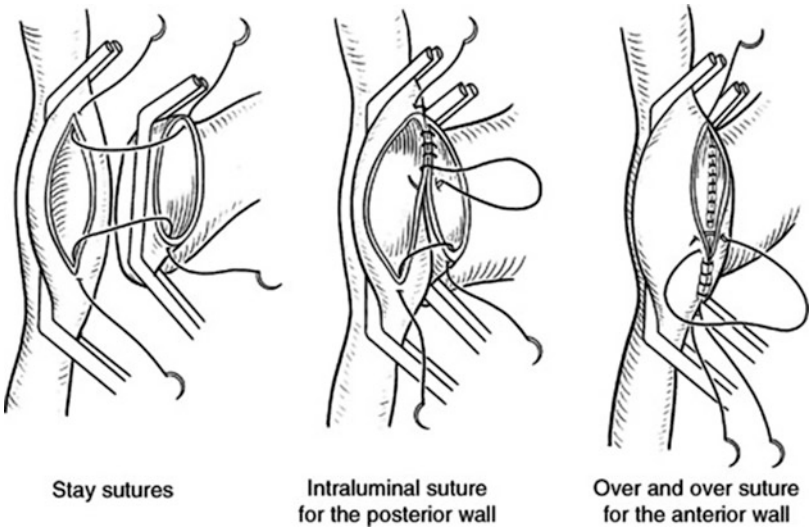

Fig. 6 End-to-side spleno-mesenteric anastomosis

When the confluence of the PV and SMV is involved, vascular clamps are placed on the splenic vein (SV), the SMV, and the PV, and the involved segment is resected (Fig. 2d). End-to-end anastomosis of the SMV and PV is first performed, a vascular clamp is placed longitudinally on the left lateral aspect of the reconstructed SMV, and end-to-side anastomosis of the SV and SMV is performed using the intraluminal suture technique for the posterior wall and the over-and-over suture technique for the anterior wall (Fig. 6). If venous congestion is not observed in the proximal organs of the SV, the division and ligation of the SV without reconstruction are a possible alternative to the abovementioned reconstruction procedure (Fig. 2d) [12]. In such cases, the formation of varices due to left-sided

portal hypertension may occur during the late phase after surgery [36, 37]. This issue will be discussed in a later chapter.

Autologous, Homologous, or Prosthetic Vein Grafting

When the resected segment of the portal vein is long and end-to-end anastomosis between the proximal and distal end cannot be performed, an autologous vein graft is interposed between the resected veins (Fig. 2e). For the autologous vein graft, the authors prefer to use the external iliac vein [32] (Fig. 7); however, other authors propose using the left renal vein [38, 39] as the autologous vein graft. There are pros and cons for both the external iliac vein and the left renal vein graft. Regarding the external iliac vein, it is possible to harvest a fairly long graft, and its diameter is almost identical to that of the PV/SMV. However, an additional skin incision is necessary to harvest the external iliac vein graft using either the intraperitoneal or extraperitoneal approach. Moreover, there is a risk of leg edema and deep venous thrombosis after harvesting an external iliac vein graft. In comparison, a left renal vein graft can be easily harvested without making a new skin incision. However, the length of the left renal vein graft is restricted by the branching of the left adrenal vein (central vein) and the gonadal vein, which should be certainly preserved to maintain venous return from the left kidney. Additionally, this type of autologous vein graft should be avoided in patients with renal dysfunction. The internal jugular vein [33, 40] and gonadal vein [41, 42] can also be used as an autologous vein graft source. The autologous vein graft should be selected considering the condition of the patients and the invaded PV/SMV. When an autologous vein graft is difficult to harvest, the parietal peritoneum [43], homologous veins [44], permanent prosthetic grafts [45], or biologic prosthetic material [46] can be used. The greatest benefit of these non-autologous vein grafts is the unlimited length of the source for

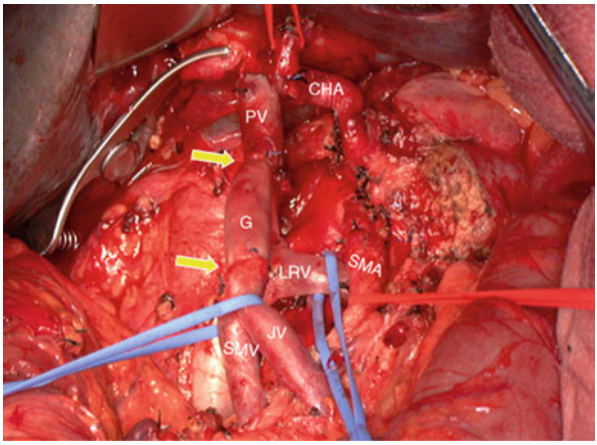

Fig. 7 Porto-mesenteric confluence resection and reconstruction using an external iliac vein graft. *PV* portal vein, *SMV* superior mesenteric vein, *JV* jejunal vein, *SMA* superior mesenteric artery, *CHA* common hepatic artery, *LRV* left renal vein, *G* external iliac vein graft. Arrows indicate the proximal and distal anastomosis of the interposed iliac vein graft. Major shunt route following splenic vein (*SV*) dissection

interposition. It should be noted, however, that the risk of thrombus formation may generally increase with the use of a non-autologous vein graft.

Vascular interposition may require a longer repair time compared with direct end-to-end anastomosis or vascular patch graft. If more than 30 min clamping is anticipated for venous reconstruction, the mesenteric flow should be bypassed to either the portal venous flow via the round ligament or systemic circulation via the greater saphenous vein to avoid severe congestion and/or ischemia of the small and large intestine [47].

Development of Left-Sided Portal Hypertension After Splenic Vein Ligation

When pancreatic head cancer invades the confluence of the SV and SMV, dissection of the SV is necessary. The SV may be reimplanted into the reconstructed SMV-PV system, but the ligation of the SV without reimplantation may also be acceptable. In such cases, the risk of left-sided portal hypertension is a controversial problem. Rosado et al. analyzed 15 patients who underwent extended pancreatoduodenectomy with PV ligation and aimed to identify postoperative venous collateral patterns and sequelae of SV ligation [48]. In all patients, the junction of the inferior mesenteric vein (IMV) with the SV or SMV was resected. In most patients (14 out of 15), a collateral route developed from the residual SV to the SMV via collateral veins in the omentum and along the colon (an inferior route; Fig. 8). At the same time, 10 out of 15 patients developed a collateral route from the residual SV to the PV via the gastric, perigastric, and coronary veins (a superior route). There were no patients who developed gastrointestinal bleeding within the study period of at least 5 months. Mean platelet count and spleen size were also unaffected. Misuta et al. reported similar surgical outcomes in 29 patients who underwent pancreatoduodenectomy with SV division [49]. They proposed that it is important to preserve the omentum [48] and transverse and right colic marginal veins to avoid the formation of varices [36]. Ligation or embolization of the splenic artery [37, 50] or anastomosis of the splenic vein to the IMV [51, 52] is another option for reducing the incidence of varices. It should be noted, however, that other reports show that the preservation of the IMV is not effective for preventing left-sided portal hypertension [53].

Complications Related to PV/SMV Resection and Reconstruction

Portal venous thrombosis is one of the most common complications following pancreatoduodenectomy with PV/SMV resection and reconstruction. The reported incidence of thrombosis following PV/SMV resection and reconstruction is 20–30% [34, 54–56], and the rate of thrombosis is lower in cases of primary end-to-end anastomosis or transverse venorrhaphy compared with interposition graft or patch venoplasty [57]. Regardless of the reconstruction method, patients who undergo preoperative chemotherapy or radiation therapy or have prolonged operative times

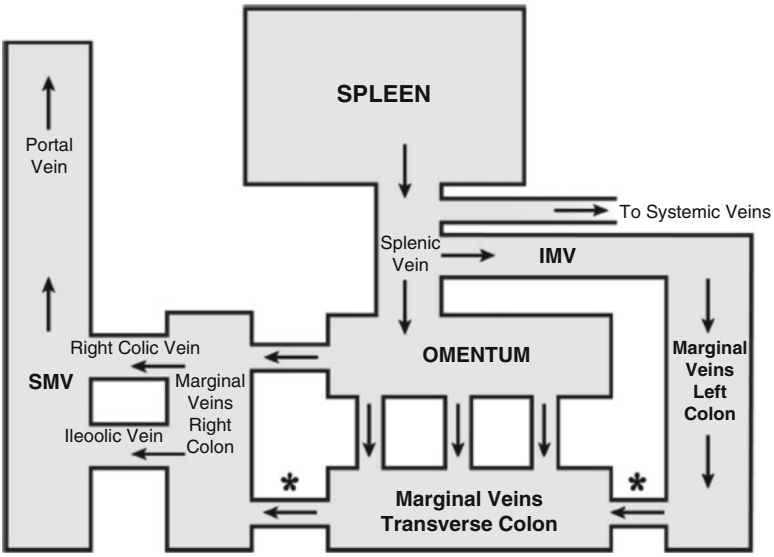

Fig. 8 Possible inferior collateral routes for decompressing the SV after ligation in an extended pancreatoduodenectomy. The SV may decompress inferiorly into the inferior mesenteric vein (*IMV*), into the omentum, or, more rarely, into the systemic circulation. Blood will flow from the IMV through the marginal veins of the colon to the superior mesenteric vein (*SMV*). If the colonic venous arcade is incomplete at the splenic flexure (*right asterisk*), this shunt may not be operative, and blood flow in the IMV will not reverse direction, or varices will format that location. Omental vessels connect to the marginal vessels of the transverse and right colon and through them and the ileocolic and right colic vessels to the SMV. Incomplete arcade at the hepatic flexure (*left asterisk*) may also result in colonic varices. IMV may rarely decompress to systemic veins. The middle colic vein is rarely available as it is almost always divided in an extended pancreatoduodenectomy with vein resection, but if present, it may decompress the marginal veins of the transverse colon. Colonic varices may also form at other locations, e.g., the cecum, depending on the completeness of the venous marginal arcade [48]

are more likely to have portal venous thrombosis [56]. When thrombosis was identified in a reconstructed PV/SMV during the postoperative course, aggressive anticoagulation therapy may be recommended unless there is a risk of bleeding complications. However, the usefulness of routine prophylactic anticoagulation therapy is controversial because the risks of thrombosis were not different between patients with and without prophylactic anticoagulation therapy in most previous reports [55, 57, 58].

Another complication after PV/SMV resection is bleeding from the anastomotic site (or other non-anastomotic site) in the PV/SMV system following pancreatectomy. This complication may be largely induced by a pancreatic fistula, in which pancreatic juice leaking from the anastomotic site melts an adjacent structure, such as the PV/SMV [59]. It is important to actively aspirate the leaking pancreatic juice using drainage catheter. However, at the same time, the catheter may tear or sometimes puncture the PV/SMV, which may lead to massive bleeding from the

PV/SMV system. Clamping a drainage catheter may be effective to stop bleeding because the intravascular pressure in the PV/SMV is not very high (approximately 5–10 mmHg) and is much lower than the arterial pressure. However, the clamping of a drainage catheter deteriorates the pancreatic fistula, which further damages the PV/SMV wall. This dilemma can be resolved by using stent graft for PV/SMV [60, 61]. The utility of stent grafting for arterial hemorrhage after pancreatectomy is widely accepted [62, 63]. In contrast, the standard procedure for postoperative PV/SMV hemorrhage is surgical repair [64, 65] when possible. However, this method is not safe when severe intra-abdominal inflammation and adhesion occur around the PV/SMV after surgery. In such cases, the stenting technique is also a feasible therapeutic option for PV/SMV hemorrhage. Regarding the PV/SMV stenting technique, a 12-Fr sheath is inserted into the main portal venous system using a transhepatic or ileocolic approach. The bleeding point is then identified with portography through the intraportal catheter or contrast radiography through the drainage tube of the pancreatic fistula (Fig. 9a). Based on the size of the PV/SMV measured with CT scanning, an appropriate stent graft size is selected to minimize endoleak (Fig. 9b, c). When the leaking point is close to the confluence of the splenic vein, gastric vein, and/or inferior mesenteric vein, these vessels can be embolized using coils and microcoils before the stent graft is deployed. The authors recommend to use anticoagulant therapy after stent graft insertion, because low pressure and slow portal venous flow may sometimes induce thrombus formation in the portal venous system.

Surgical Results (Summary of Five Meta-Analyses of Venous Resection)

Several reports have indicated that pancreatectomy with PV/SMV resection can be performed with acceptable morbidity and mortality, and survival was comparable for patients with and without PV/SMV resection [23, 24, 33, 66]. In contrast, other reports have shown poorer survival in patients who underwent PV/SMV resection compared with those who did not [67]. Presently, there is no consensus regarding which patients with pancreatic cancer benefit from PV/SMV resection. Because the pattern of PV/SMV invasion and each surgeon's ability to intraoperatively recognize the extent of PV/SMV invasion in each surgeon are variable, it is extremely difficult to perform RCTs that elucidate a clinical benefit of PV/SMV resection in pancreatic cancer patients with vascular invasion. Therefore, at this time, a systematic review that collects a large number of reports comparing the surgical outcomes of patients with and without PV/SMV resection may be one of the best ways to clarify the clinical value of combined resection of PV/SMV in pancreatic cancer patients.

Since 2006, five meta-analyses or systematic reviews comparing the clinical outcomes of patients who underwent pancreatectomy with and without synchronous PV/SMV resection have been reported [26, 68–71] (Table 2). The study years varied among these reports, ranging from 1996 to 2014. Most of the analyses included more

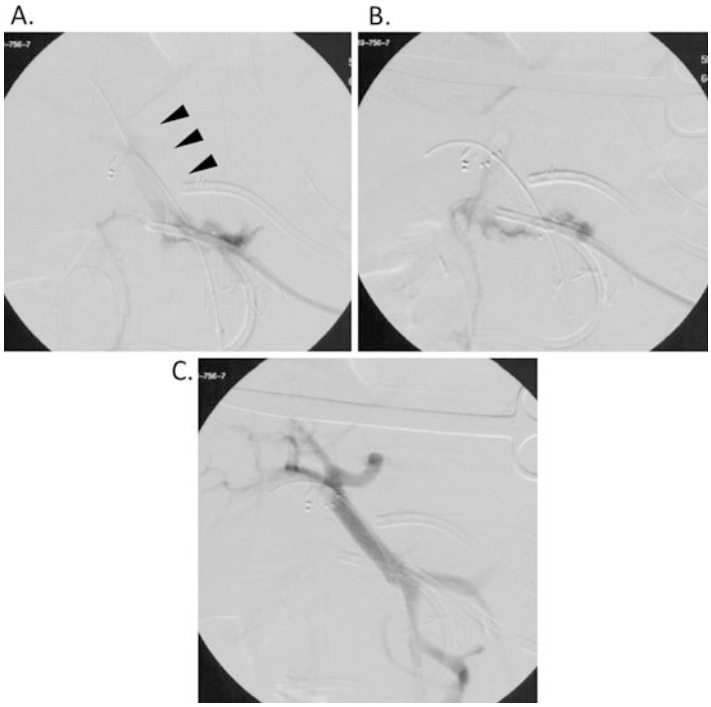

Fig. 9 Insertion of a stent graft for portal vein (*PV*) hemorrhage. (**a**) Contrast radiography from a drainage tube for pancreatic fistula. *Arrowhead*: PV was visualized. (**b**) Insertion of PV stent (diameter 10 mm, length 40 mm) through the ileocolic route. (**c**) Portal venography after PV stent insertion

than 2000 pancreatic cancer patients. The rate of vascular resection ranged from 17.6 to 44.8%.

Perioperative Outcomes

Operation time and intraoperative blood loss may increase during vascular resection. However, the differences in these factors between patients who underwent vascular resection and those who did not were acceptable. According to the meta-analysis by Siriwardana et al., the mean (range) duration of PV/SMV occlusion was 20 (7–302) minutes, and the mean (range) resected length of the PV/SMV was 3.9 (0.8–10.0) cm [26]. The overall morbidity and mortality rate after surgery were generally comparable between patients who did and did not undergo PV/SMV resection. However, the most recent meta-analysis by Giovinazzo et al. showed a higher morbidity and mortality rate in patients who underwent PV/SMV resection [70]. Nevertheless, these rates are still acceptable for this type of surgery. The incidence rate of delayed gastric emptying, which is one of the most commonly observed complications, was

Table 2 Summary of 5 meta-analyses

Authors		Siriwardana HPP	Ramacciato G	Zhou Y	Yu XZ	Giovinazzo F
Meta-analysis?		No	No	Yes	Yes	Yes
Year of publication		2006	2009	2012	2014	2016
Study year		1996–2005	2000–2008	1994–2010	1994–2013	1996–2014
Number of analyzed articles		52	12	19	22	27
Number of analyzed patients		6333	891	2247	2890	9005
Number of patients with VR (%)		1646 (24.0)	399 (44.8)	661 (29.4)	794 (27.5)	1587 (17.6)
Operating time [min]	Without VR With VR	ND 513 (168–1740)	ND (308–667)	427.6 **497.3***	854 **326***	439 **550***
Blood loss in [ml]	Without VR With VR	ND 1750 (300–26,000)	ND (700–3083)	896 **1412***	199 **128***	1316 **1921***
Time of PV/SMV occlusion [min]		20 (7–302)	(8–40)	ND	ND	ND
Length of resected PV/SMV [cm]		3.9 (0.8–10)	(1.5–5.0)	ND	ND	ND
Morbidity rate [%]	Without VR With VR	ND 42 (9–78)	34.5 (16.7–54) ND	44.0 41.9	37.2 38.4	32.1 **38.6***

(*continued*)

Table 2 (continued)

Authors		Siriwardana HPP	Ramacciato G	Zhou Y	Yu XZ	Giovinazzo F
Mortality [%]	Without VR With VR	ND 5.9* (0–33)	2.9 (0–7.7) ND	3.7 3.3	4.3 5.5	3.0 **3.9***
Delayed gastric emptying [%]	Without VR With VR	ND ND	ND ND	16.4 18.8	ND ND	8.4 11.0
Pancreatic fistula [%]	Without VR With VR	ND ND	ND ND	13.1 **9.0***	11.7 **7.2***	11.4 11.5
Postoperative hospital stay [days]		21 (7–283)	(12–68.8)	ND	ND	ND
Histological PV/SMV invasion [%]		63.4	63.9 (42.9–100)	56.9 (21–100)	ND	61 (22–83)
Positive resection margin [%]	Without VR With VR	ND 39.8	ND 33.8 (8.3–83.4)	ND ND	24.0 **32.0***	31.0 **37.0***
Nodal metastasis [%]	Without VR With VR	ND 67.4	ND 71.1 (33.3–97)	ND ND	64.8 **69.0***	54.6 62.1
Median survival [m]	Without VR With VR	ND 13 (1–109)	ND (13–22)	ND ND	ND ND	19.5 14.3

1-year survival [%]	Without VR With VR	ND 50	ND (31–83)	61.8 61.3	58.7 53.7	Worse in patients with VR
3-year survival [%]	Without VR With VR	ND 16	ND ND	26.6 19.4	19.2 13.7	Worse in patients with VR
5-year survival [%]	Without VR With VR	ND 7	(9–18) ND	17.0 12.3	3.7 **10.1***	Worse in patients with VR
Major message		The high rate of nodal metastases and low 5-year survival rates. By the time of tumor involvement of the portal vein cure is unlikely, even with radical resection	Selected articles only pancreatic cancer with PV/SMV resection. No difference in the complication rate	PV/SMV resection is justified because it can result in good perioperative outcome and long-term survival comparable to that without PV/SMV resection	Equal perioperative morbidity and mortality. R0 resection is important for survival	Increased postoperative mortality, higher rates of non-curative resection, and worse survival after surgery in patients with PV/SMV resection

Number in the parenthesis indicates range
ND not described
VR, PV, and/or SMV resection
*$p < 0.05$ versus without VR

comparable between the two groups. In contrast, the risks of pancreatic fistula were lower in patients who underwent PV/SMV resection, probably because of greater fibrotic change of the remnant pancreas resulting from the obstruction or stenosis of the main pancreatic duct.

Pathological Positivity

Patients who underwent PV/SMV resection were more likely to have a positive resection margin than patients who did not undergo venous resection. Histological evidence of PV/SMV invasion was detected in approximately 60%. In other words, in 40% of patients who underwent PV/SMV resection, the PV/SMV was not affected by the cancer but instead was probably involved in the inflammatory response of the tissue surrounding the cancer. The resection margin was positive in more than 30% of patients not only in the resected part of the PV/SMV but also in the other dissected pancreatic margins. Nodal involvement was observed in 60% to 70% of patients. Patients with PV/SMV invasion tend to have more negative pathological prognostic factors, such as positive lymph node metastasis and positive resection margins. These results indicated that when pancreatic cancer grows outside of the pancreas and reaches to PV/SMV, it is difficult to thoroughly eradicate tumor cells, even by performing radical surgery with PV/SMV resection. These clinical observations are supported by the immunohistochemical analysis of specimens from pancreatic head cancers that have invaded the PV/SMV. It was demonstrated that histological tumor invasion of the PV/SMV is characterized by aggressive biology and stromal fibroblast activation through a loss of membranous E-cadherin in tumor buds, increased vimentin expression, and activated cancer-associated fibroblasts (CAFs) [72].

Survival

According to the first large systematic review, conducted by Siriwardana et al., the median (range) survival was 13 (1–109) months for 917 patients who underwent PV/SMV resection in 31 studies [26]. The survival of patients who underwent PV/SMV resection was clearly inferior to that of patients who did not undergo PV/SMV resection. However, we should be cautious about the time frame (since 1966 to 2006) and heterogeneous study cohorts included in this meta-analysis. In fact, a more recent meta-analysis by Zhou et al. involving 19 studies (from 1994 to 2010) of pancreatectomies for pancreatic cancer and comparing 661 patients who underwent PV/SMV resection and 2247 patients who did not indicated that pancreatectomy combined with PV/SMV resection for pancreatic cancer is justified because it can have good perioperative outcomes and its long-term survival is comparable to that obtained with standard resection [68]. The meta-analysis by Yu et al. also showed compatible survival outcomes between patients with and without PV/SMV resection [69]. Other recent cohort studies also support the clinical

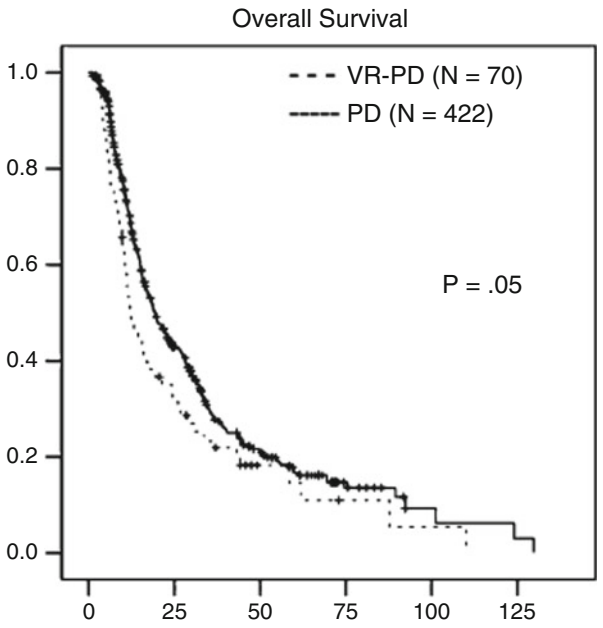

Fig. 10 Overall survival in patients undergoing pancreaticoduodenectomy with and without vein resection. *PVR-PD* vein resection combined with pancreaticoduodenectomy, *PD* pancreaticoduodenectomy without vein resection [75]

relevancy of combined PV/SMV resection for pancreatic cancer [71, 73–75]. In particular, the two largest modern multi-institutional series examining patients with or without isolated vein involvement in pancreatic cancer, conducted in the United States [75] and the United Kingdom [66], found that the oncological outcome did not differ between the patients with and without vein involvement (Fig. 10). It also should be noted, however, that the most recent meta-analysis, by Giovinazzo et al., indicated that patients with PV/SMV resection not only showed worse survival after surgery but also had higher rates of postoperative mortality and non-curative resection [70]. Taken together, the clinical benefit of PV/SMV resection for pancreatic cancer remains controversial.

Conclusion

Conflicting statements regarding the role of surgical resection of the PV/SMV during pancreatoduodenectomy have been made, and no RCTs have been conducted to clarify the surgical value of concomitant PV/SMV resection. In general, pancreatoduodenectomy with PV/SMV resection and reconstruction was not associated with increased morbidity and mortality and provided a negative surgical margin, and patients had a better survival than unresected patients. Therefore, combined PV/SMV resection and reconstruction during pancreatoduodenectomy should always be considered as an effective treatment modality for patients with pancreatic cancer adherent to the PV/SMV system in the absence of other contraindications for resection.

Key Practice Points

- The use of combined portal vein resection and reconstruction in pancreatoduodenectomy is determined preoperatively according to CT, MR, and/or portography findings.
- Intraoperatively, the type of portal vein resection and reconstruction is based on the degree of local cancer invasion of the vein.
- The types of resection and reconstruction are as follows:
  1. Wedge resection
     - Direct transverse suture
     - Patch closure
  2. Segmental resection
     - Direct end-to-end anastomosis
     - Segmental autologous vein grafting
- When the SMV/PV resection and reconstruction are performed with the ligation and division of the SV, there is a possibility that postoperative varices in the colon and esophagus will develop due to left-sided portal hypertension. Preservation of the omentum and transverse and right colic marginal vein is essential to avoid the formation of varices.
- Common complications after SMV/PV resection are thrombosis, stenosis, and hemorrhage. Thrombosis can be treated surgically and/or with anticoagulant therapy. To avoid stenosis after reconstruction, the threads used for the venous reconstruction should be loosely tied after the sufficient expansion of the anastomosed vessel with blood flow. Hemorrhage from the SMV/PV reconstruction can be treated using a full-coverage stent graft inserted via the transhepatic or ileocolic route.

Future Research Directions

- Randomized control trials are necessary to clarify the value of portal vein resection in pancreatoduodenectomy for pancreatic cancer.
- Reevaluation of the accuracy of preoperative diagnoses of portal vein invasion should be performed using modern diagnostic modalities to clarify preoperative indications for combined portal vein resection in pancreatoduodenectomy for ductal adenocarcinoma of the pancreatic head.

Cross-References

► Arterial Resection in Pancreatic Cancer
► Interventional Radiology for Pancreatic Cancer
► Staging and Postoperative Outcomes Using the International Study Group of Pancreatic Surgery (ISGPS) Classifications
► Surgical Resection for Pancreatic Cancer Using the International Study Group of Pancreatic Surgery (ISGPS) Classifications

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Controversies in Pathology Reporting and Staging

Fiona Campbell and Caroline Sophie Verbeke

Contents

Abstract

Following surgery for pancreatic cancer, it is the histopathologist who examines, dissects, and samples the resection specimen for microscopic (histologic) assessment, with the aim of producing a final pathology report that includes all the relevant prognostic information and accurate tumor staging. However, there is no universally agreed pathology protocol for the handling and sampling of pancreatic cancer resection specimens, particularly pancreatoduodenectomy specimens, and pathologists have differing opinions over what is a resection margin and

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when it should be considered involved. The increasing use of neoadjuvant therapy has also led to new challenges for the pathologist. Differences in interpretation of the TNM staging system can mean that two pathologists stage the same pancreatic cancer resection specimen quite differently. This chapter discusses the pathology reporting and staging of pancreatic cancer resection specimens, with particular emphasis on the challenges and areas of controversy for the pathologist.

Keywords
Pathology · Pancreas · Cancer · Margin · Staging · Tumor regression · Neoadjuvant therapy

Introduction

The number of pancreatic resection specimens received by the pathologist has increased exponentially over the last 20 years [1]. Not all resections, however, are performed for malignancy, and, therefore, the pathologist plays an important role in establishing the correct diagnosis. Following surgery for pancreatic cancer, it is the histopathologist who examines, dissects, and samples the resection specimen for microscopic (histologic) assessment, with the aim of producing a final pathology report that includes all the relevant prognostic information and accurate tumor staging. However, there is no universally agreed pathology protocol for the handling and sampling of pancreatic cancer resection specimens, particularly pancreatoduodenectomy specimens, and pathologists have differing opinions over what is a resection margin and when it should be considered involved. There are also differences of opinion, between pathologists, in interpretation of the current AJCC/UICC TNM staging system [2, 3], which may influence future management of patients and entry into clinical trials. These differences in pathology opinion can also influence the outcome of studies on prognostic factors in pancreatic cancer, which, in turn, can prevent meaningful comparison of different studies.

This chapter will discuss the reporting and staging of resection specimens for pancreatic cancer, mainly pancreatic ductal adenocarcinoma (PDAC) and its variants, as classified by WHO 2010 [4]. The term "pancreatic cancer", however, is often used synonymously with "periampullary cancer" to denote any tumor arising in the head of the pancreas. Cancers in the head of the pancreas may arise from the duodenum, ampulla of Vater, distal bile duct, or pancreas. Distinction between these entities is important because of their differing TNM staging and prognosis, as well as their management and the entry of patients into clinical trials [2, 3]. Accurate distinction between these different cancers is also crucial for identifying possible differences in their epidemiology, etiology, and molecular biology. Errors in determining the primary origin of the tumor are highly likely to be one explanation for the considerable variation in R1 resection rates for pancreatic ductal adenocarcinoma (PDAC) in the literature. The R1 rate for true ampullary cancers is much less

than that for PDAC, while the R1 rate for distal bile duct cancer approaches that of PDAC [5–8]. Expert review of pancreatic head tumors diagnosed as PDAC has highlighted the issue of correctly establishing the primary origin of tumors in the head of the pancreas: in one review, 23% of cancers reported as PDAC were in fact carcinomas of the ampulla of Vater or of the bile duct [9].

Reported incomplete (R1) resection rates for PDAC vary considerably in the literature from 8% to 85% [10, 11]. As will become apparent from the following chapter discussion, the reasons for these different R1 rates can be multifactorial. R1 rates may be influenced by differences in specimen dissection techniques [12], differing opinions over what constitutes a resection margin, adequacy (or not) of margin sampling, definition of microscopic margin involvement [13], and, as indicated above, erroneous inclusion of primary cancers of the ampulla of Vater or distal common bile duct in studies of PDAC [9]. When a standardized pathology examination protocol is used with an agreed microscopic definition of R1, then microscopic margin involvement is a common finding in PDAC and is found to be of prognostic significance in many, if not all, studies [11, 14–16]. The high rates of R1 resections in PDAC are not a marker of low-quality surgery, but rather an indicator of high-quality pathology [14]. There are many challenges and areas of controversy for the pathologist when reporting and staging pancreatic cancer resections, including those arising from an increase in the number of resections following neoadjuvant therapy. The following sections will discuss these challenges and areas of controversy in more detail.

Dissection

The importance of the macroscopic examination and dissection of the pancreatic resection specimen (particularly the pancreatoduodenectomy specimen), by the pathologist, cannot be overemphasized [17]. The primary origin of the cancer (i.e., pancreas vs. ampulla vs. bile duct vs. duodenum), its size and extent, lymph node status, and margin status influence the TNM and R classification/staging, which, in turn, may determine further therapy and/or entry into clinical trials. There is currently no internationally accepted standardized pathology dissection protocol [7], leading to considerable variation in the reporting of factors that are of potential clinical and prognostic significance [12, 13, 18, 19].

For many years, pathologists examined the pancreatoduodenectomy specimen by inserting probes into the common bile duct and main pancreatic duct and then slicing the head of the pancreas along these probes [20]. Probing the pancreatic duct can be difficult, particularly in the distal portion, where it can be kinked, and because of its narrow bore. With increasing numbers of pancreatic resections being performed over the last 20 years [1], more pathologists have encountered these specimens and developed their own methods of dissection without the need to probe the ducts.

The main dissection approaches being used currently include bivalving or multivalving, bread loafing, and the axial slicing techniques [21–23].

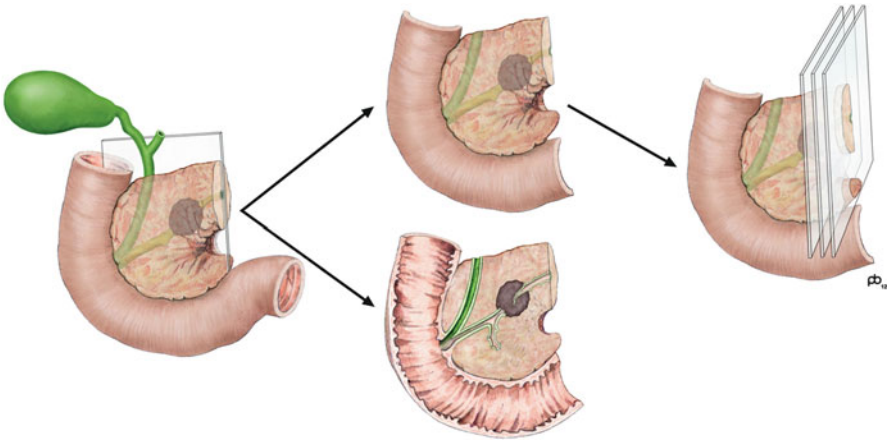

Fig. 1 In the bivalving or multivalving technique, the pancreatoduodenectomy specimen is sliced in a plane defined by probes placed in the main pancreatic duct and the common bile duct

Bivalving or Multivalving

In this technique, the main pancreatic duct and common bile duct are probed, and the specimen is sliced once (bivalving) or several times (multivalving) along the plane defined by both probes (Fig. 1). This approach can be technically difficult if one or both ducts are distorted or obstructed by tumor. Subsequent slicing of the specimen along the probes may also be challenging. Advocates of this method find it particularly helpful in the demonstration and assessment of primary ampullary tumors [24].

Bread Loaf Slicing

In the bread loaf slicing technique, the pancreatic head is serially sliced along a plane perpendicular to the longitudinal axis of the pancreatic neck (Fig. 2). With this technique, dissection of the periampullary region can be suboptimal, because the descending part of the duodenum is sliced longitudinally [17].

Axial Slicing

In this technique, the pancreatic head is serially sliced in the axial plane that is perpendicular to the descending part of the duodenum (Fig. 3). This dissection plane is identical to that of radiological imaging, i.e., computerized tomography scanning or magnetic resonance imaging, allowing correlation between radiology and pathology.

The axial slicing technique is easy to perform and can be used for all pancreatoduodenectomy specimens, regardless of the pathology encountered [17]. The

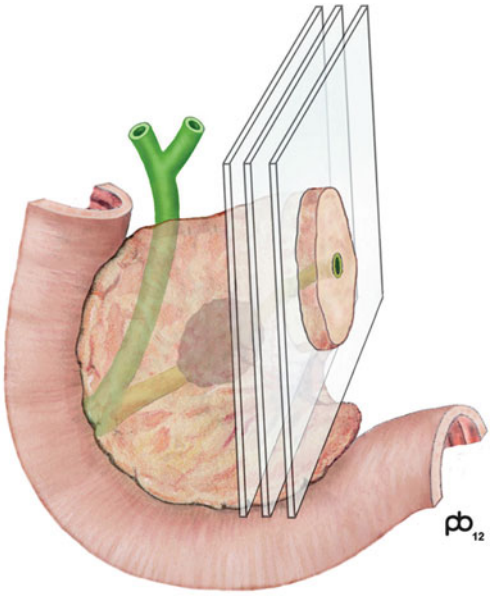

Fig. 2 In the bread loaf slicing technique, the pancreatoduodenectomy specimen is serially sliced in a plane perpendicular to the pancreatic neck

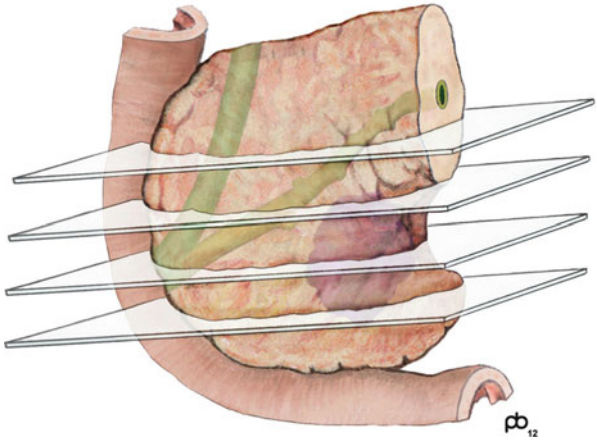

Fig. 3 In the axial slicing technique, the pancreatoduodenectomy specimen is serially sliced in a plane perpendicular to the long axis of the descending duodenum

pancreatic surface remains intact, facilitating margin assessment, and the main dissection plane is independent of the configuration of the main pancreatic duct and common bile duct, which influences the bivalving or multivalving technique. Other advantages of the axial slicing technique include the fact that the key anatomic structures (ampulla of Vater, common bile duct, main pancreatic duct) always occur at the same position in the specimen slices (allowing identification of anatomic variation and pathologic lesions), and the entire circumferential surface of the pancreas is present in each specimen slice (enabling accurate margin assessment along the entire craniocaudal length of the pancreatic head) [17].

Distal pancreatectomy specimens can be serially sliced in the sagittal plane, i.e., perpendicular to the longitudinal axis of the pancreas. This avoids the disruption of the specimen surface if the main pancreatic duct is opened longitudinally. Total pancreatectomy specimens can be dissected by a combination of axial slicing of the head and sagittal slicing of the body and tail [2].

The axial slicing technique advocated by the Japanese Pancreas Society [23] involves serially slicing the specimen perpendicular to an axis that follows the curvature of the pancreatic head. This has the disadvantage for the pathologist of producing slices that are wedge-shaped rather than uniform slices [17].

Macroscopic Assessment and Sampling

The pancreatic resection specimen can be examined fresh (e.g., for biobanking), following fixation in formalin. Prior to dissection, the different surfaces of the pancreas should be inked (according to a locally agreed color code) to facilitate identification of these surfaces during macroscopic and microscopic examination [21]. The dimensions of the pancreas, duodenum, stomach, extrapancreatic common bile duct, and other structures, such as the gallbladder or attached portion of superior mesenteric vein or portal vein, should be measured and recorded. Following dissection, the serial specimen slices can be laid out in sequential order and photographed to provide a permanent record of the gross findings. These images can be extremely helpful when interpreting the microscopic findings and when discussing the pathologic findings at clinical meetings such as the multidisciplinary team meeting.

After describing the gross appearance of the specimen, tumor, and any other pathology, tissue samples should be taken. Tissue sampling should be extensive because pancreatic ductal adenocarcinoma is highly infiltrative and invades much more widely than can be appreciated by the naked eye. It can also be difficult to distinguish carcinoma from chronic pancreatitis. The size and extent of the tumor are often underestimated on the gross examination. There is also a significant correlation between the number of tissue blocks taken and the likelihood of identifying a positive resection margin [11, 25].

The tumor should be sampled en bloc with adjacent structures and circumferential surfaces/margins, together with all lymph nodes, transection margins (pancreatic neck, common bile duct, duodenum or stomach, jejunum), the gallbladder (if present), other organs (e.g., spleen in the distal pancreatectomy), and background tissue. When a segment or sleeve of portal vein or superior mesenteric vein is present, this is best sampled en bloc with the adjacent superior mesenteric vessel groove and pancreas, to assess for tumor invasion of the vein wall. It is recommended that the entire resected vein is embedded to determine whether its adherence is due to tumor infiltration of the vessel wall or due to inflammation and fibrosis [26, 27]. When an attached segment of vein is not adherent to the superior mesenteric vessel groove over its entire length, then both cut ends (i.e., transection margins) can be sampled separately as en face tissue slices [21].

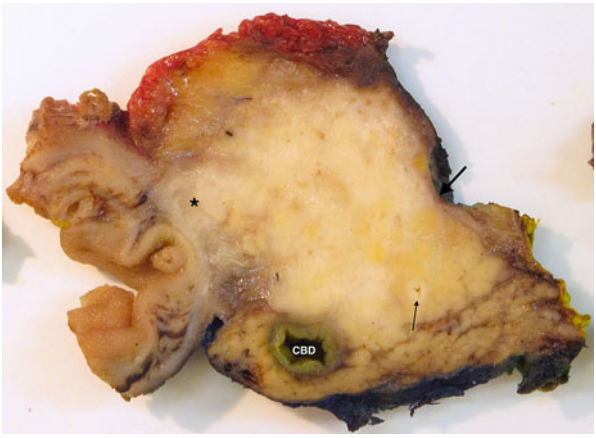

Fig. 4 Axial specimen slice with a large PDAC that infiltrates the duodenal wall (*asterix*) and main pancreatic duct (*thin arrow*), extends close to the groove of the superior mesenteric vein (*thick arrow*), but spares the common bile duct (*CBD*)

Tumor Origin

The exact location of the tumor with respect to the bile duct, ampulla, and duodenum is crucial for establishing the primary origin of the cancer (Fig. 4). However, the precise origin of a tumor may be difficult to determine, particularly when the tumor is large and involves more than one potential site of origin. The localization of the center of the tumor is the most helpful and important diagnostic criterion. This is assessed macroscopically and confirmed microscopically [19]. The presence of neoplastic precursor lesions, e.g., adenoma of the ampulla, may be helpful in identifying the primary origin of the tumor, but precursor neoplasia of the bile duct is much less commonly observed in association with bile duct cancer and usually presents as flat dysplasia rather than as an adenomatous polypoid lesion. Pancreatic intraepithelial neoplasia (PanIN) as evidence of a pancreatic origin cannot be relied upon, since it can be seen in the background pancreas of specimens with ampullary or bile duct cancer, as well as pancreatic cancer [28, 29]. In addition, cancerization of background structures (e.g., ducts or duodenal mucosa) can mimic dysplasia [30]. Although immunohistochemistry may be helpful in distinguishing intestinal-type carcinomas from pancreatobiliary-type carcinomas arising in the ampulla of Vater [31], there are currently no immunohistochemical markers that can distinguish between pancreatobiliary-type carcinomas of the ampulla and PDAC or bile duct carcinoma. Moreover, PDAC can have an intestinal morphology, thus mimicking duodenal or ampullary carcinomas [30].

Margins

Assessment of the margin status in pancreatic cancer resection specimens is the source of much controversy. Pathologists differ in what they consider to be a margin (and thus whether or not it should be sampled) and when a margin should be

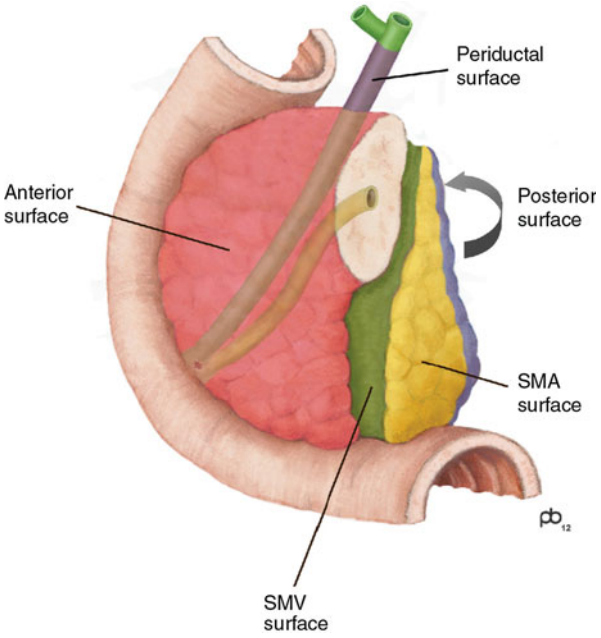

Fig. 5 The circumferential margins of a pancreatoduodenectomy specimen. The different surfaces can be inked according to a locally agreed color code

considered involved (R1) or not (R0) [13]. While all pathologists agree that the transection margins of the pancreatic neck, common bile duct, jejunum and proximal duodenum, or stomach should be sampled and evaluated, practice varies when it comes to examination of the "circumferential margins" of the pancreas. The circumferential margin of the head of the pancreas includes the anterior surface, the posterior surface, the superior mesenteric vein (SMV) groove, and the superior mesenteric artery (SMA) margin (Fig. 5). Pathologists differ in the terminology they use for the SMA margin. It is also referred to as the "retroperitoneal margin" (but the whole of the pancreas is retroperitoneal), "uncinate margin", "medial margin", "radial margin", "mesenteric margin", "mesopancreatic margin", and "posterior margin". Use of the latter term ("posterior margin") for the SMA margin means that comparisons of different published studies may not be valid if others are using the term to refer to the true posterior surface of the pancreatic head. A further circumferential margin that should also be considered in pancreatoduodenectomy specimens is the connective tissue sheath that surrounds the extrapancreatic common bile duct. This thin layer of tissue may be invaded by carcinomas arising in the extrapancreatic common bile duct or by infiltration from carcinomas of the intrapancreatic common bile duct or PDAC arising in the cranial part of the pancreatic head.

The only circumferential margin considered to be a resection margin (and therefore sampled) by some pathologists, particularly those following an American protocol [32], is the SMA margin [33, 34]. Tumor involvement of the so-called "dissection or mobilization" margins (i.e., posterior margin, SMV groove, and

around the extrapancreatic common bile duct), however, does affect survival, although it has been suggested that the influence on survival may be less than that when transection margins are involved [16]. The anterior surface is an anatomical surface rather than a true resection margin, but tumor involvement of this surface is associated with increased risk of recurrence [35], and, therefore, this surface should also be sampled and evaluated. Many current pathology guidelines now highlight the importance of evaluating all of the circumferential margins/surfaces as well as the transection margins [26, 36, 37]. In most standardized studies, the superior mesenteric vessel (medial) margin and the posterior margin are the two most commonly involved by tumor [11, 12, 14–16].

PDAC has a highly infiltrative growth pattern and often extends much further than apparent on macroscopic (gross) examination. Therefore, to assess margin status, extensive sampling of the margins should be undertaken. It has been shown that there is a significant correlation between an increasing number of tissue blocks taken from the resection specimen and an increasing likelihood of an R1 classification [11, 25]. It has also been shown that the method of dissection of pancreatoduodenectomy specimens influences R1 rates [12]. This meta-analysis found a pooled R0 rate of 29% in studies using an axial slicing technique and a definition of R1 $<$ 1 mm, while studies using other techniques and R1 $<$ 1 mm had a pooled R0 rate of 49% [12].

Completeness of excision should be assessed macroscopically and then be confirmed (or not) by microscopy. But what constitutes a microscopic complete (R0) resection or incomplete (R1) resection? The UICC TNM residual tumor (R) classification considers a resection margin involved when there is either macroscopic (R2) or microscopic (R1) transected tumor directly at a surgical resection margin (i.e., 0 mm clearance) [3]. In 2002, the Royal College of Pathologists, UK, pancreas dataset adopted the "1 mm rule" from the guidelines for reporting rectal cancer and considered PDAC within 1 mm of a margin to be an R1 resection [36]. A clearance of $<$1 mm had been shown to be associated with an increased risk of local recurrence in rectal cancer [38, 39]. However, similar studies have not been undertaken for pancreatic cancer. The growth pattern of PDAC is highly infiltrative and discontinuous, unlike that of rectal cancer. The distances between tumor cells in PDAC become significantly greater in the periphery of the tumor compared to the center. This is in contrast with colorectal cancer where there is no difference in the intercellular distances between tumor cells within the different regions of the tumor [40]. Reflecting this widely dispersed growth pattern for PDAC, others have suggested that a minimum of 1.5 mm or 2 mm clearance should be applied for an R0 resection [41–43]. These studies showed that a margin clearance of $<$1.5 mm or 2 mm was associated with a long-term survival equivalent to that of patients with directly involved (0 mm clearance) margins.

There is increasing agreement that a clearance of 0 mm is not appropriate for PDAC, but the distance required remains unknown. Current pathology guidelines [26, 36] continue to use the 1 mm rule, which has now been adopted by AJCC TNM 8 (but not UICC TNM) [44]. However, this 1 mm rule probably underestimates the presence of microscopic residual disease. It is probably more important that the

pathologist's report includes the exact measurement of the distance from the tumor to the nearest margin, so that it is clear why the resection has been considered R0 or R1.

In about 7% of pancreatic cancer resection specimens, there is no direct tumor involvement of a margin, but tumor is found at a margin within a lymph node, vascular channel, or perineural cleft [14]. One UICC TNM communication has stated that if tumor is attached to the lumen of the vessel wall or invades the vessel wall at the margin, then this should be classified as R1, but does not comment on whether lymph node or perineural involvement at a margin should also be classified as R1 [45]. Many pathologists would consider nodal or perineural deposits at a margin to be R1 [13], and some current pathology guidelines have recommended that such vessel, nodal, or perineural margin involvement is considered R1, with the caveat that this mode of margin involvement should be clearly stated as the reason for calling a resection specimen R1 in the histology report [26, 36]. However, these three modes of tumor spread are independent biological processes, recorded separately in histology reports and as optional descriptors in TNM (pV, pL, pPn), and can influence the risk of residual tumor in the patient whether or not they occur in proximity to a resection margin. For this reason, many pathologists would not consider their presence at a margin as R1, arguing that it is not appropriate to duplicate their biological risk by also classifying them as R1 [13].

Lymph Nodes

All lymph nodes present in a resection specimen should be sampled (each in its entirety, unless metastasis is seen macroscopically) by the pathologist. Lymph nodes are present in the peripancreatic tissue and the tissue surrounding the extrapancreatic common bile duct. In pancreatoduodenectomy specimens, lymph nodes may also be present in the infra-gastric and perigastric fat. Lymph nodes can be allocated to different lymph node stations using the Japanese Pancreas Society or UICC systems [3, 23]. The JPS system is much more detailed than the UICC system and allocates the lymph nodes to a larger number of different stations. The JPS system also includes distant lymph node stations, as well as regional lymph nodes [23]. Lymph nodes around the common hepatic artery are not specifically stated as regional for the pancreas in UICC TNM 7 [3], but are considered to be regional lymph nodes in AJCC TNM 7 and 8 [2, 44] and the JPS system [23]. UICC TNM 8 [46] now clearly states that common hepatic artery lymph nodes are regional, thereby removing the potential for the classification of a common hepatic artery lymph node as a distant metastasis. This anatomic division of regional lymph nodes is not necessary for TNM staging, but can act as an aide memoire to help the pathologist to locate the peripancreatic lymph nodes.

In the axial slicing technique, the lymph nodes are sampled en bloc with the adjacent pancreas and circumferential margins/surfaces. Dissection of lymph nodes from the peripancreatic fat, including by the "orange peel" method [47], prior to slicing the main specimen disrupts the specimen surface, precluding accurate margin assessment and an accurate measurement of the distance from the tumor to the

adjacent circumferential resection margin/surface. It also precludes assessment of direct tumor invasion into a lymph node (see Sect. 7). When using the axial slicing technique, careful consideration of the shape, size, and location of a lymph node in both the microscopic section and the close-up photographs of the corresponding specimen slices can help to avoid counting the same lymph node more than once.

TNM Classification/Staging

Pathologic staging (pTNM) of pancreatic ductal adenocarcinoma following resection gives an indication of the extent of the cancer and prognosis for long-term survival [48]. The two TNM classification systems used for staging are those of the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC) [2, 3]. These two systems, however, do not always concur (e.g., pN staging for cancers of the ampulla of Vater in TNM 8) [44, 46].

In the currently used AJCC/UICC TNM 7 [3], the pT stage requires (macroscopic and microscopic) pathologic assessment of the size of the tumor and whether it is limited or not to the pancreas (Table 1). However, pathologists differ in their interpretation of the pT staging (Table 2).

A pT1 pancreatic ductal adenocarcinoma is limited to the pancreas and 2 cm or less in greatest dimension [2, 3]. The increasing number of resections of intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs), however, has led to the detection of small, "early" pancreatic ductal adenocarcinomas much less than 2 cm in size. This led to a proposal that pT1 pancreatic cancers should be substaged into pT1a, pT1b, and pT1c according to whether the size was $\leq$0.5 cm, $>$0.5 cm and $\leq$1 cm, and $>$1 cm and $\leq$2 cm, respectively [49, 50]. This proposal has now been accepted by AJCC and UICC in TNM 8 [44, 46].

In AJCC/UICC TNM 7, pT1 and pT2 pancreatic cancers are confined to the pancreas, whereas pT3 cancers extend beyond the pancreas but without involvement of the celiac axis or superior mesenteric artery (see Table 1) [2, 3]. The pancreas, however, does not have a capsule, and it is not always easy for the pathologist to decide what is peripancreatic tissue; the distinction between pancreas and peripancreatic soft tissue is often obscured by (chronic pancreatitis or tumor-related) fibrosis or fatty replacement [21]. There is also controversy over the pT staging of pancreatic ductal adenocarcinomas that invade the intrapancreatic common bile duct, which is a common event, even with small tumors. In UICC TNM 5, pT3 was defined as "tumor extends directly into any of the following: duodenum, bile duct, peripancreatic tissues" with the footnote that "direct invasion to bile ducts and duodenum includes involvement of the ampulla of Vater" [51]. Although some guidelines (e.g., those of the College of American Pathologists) [32] clearly state that bile duct involvement only refers to involvement of the extrapancreatic bile duct, many pathologists would consider involvement of the intrapancreatic bile duct as extension beyond the pancreas and, therefore, stage such tumors as pT3 in TNM 7 (Table 2).

Table 1 TNM staging of pancreatic cancer according to AJCC/UICC TNM 7 and 8 [2, 3, 44, 46]

AJCC/UICC TNM 7				**AJCC/UICC TNM 8**			
T1 – tumor limited to pancreas, 2 cm or less in greatest dimension				T1 – tumor 2 cm or less in greatest dimension			
				T1a – tumor 0.5 cm or less in greatest dimension			
				T1b – tumor greater than 0.5 cm and less than 1 cm in greatest dimension			
				T1c – tumor greater than 1 cm but no more than 2 cm in greatest dimension			
T2 – tumor limited to pancreas, more than 2 cm in greatest dimension				T2 – tumor more than 2 cm but no more than 4 cm in greatest dimension			
T3 – tumor extends beyond pancreas, but without involvement of celiac axis or superior mesenteric artery (SMA)				T3 – tumor more than 4 cm in greatest dimension			
T4 – tumor involves celiac axis or SMA				T4 – tumor involves celiac axis, SMA, and/or common hepatic artery			
N0 – no regional lymph node metastasis				N0 – no regional lymph node metastasis			
N1 – regional lymph node metastasis				N1 – metastasis in 1–3 regional lymph nodes			
				N2 – metastasis in 4 or more regional lymph nodes			
TNM 7 stage grouping				**TNM 8 stage grouping**			
Stage 0	Tis	N0	M0	Stage 0	Tis	N0	M0
Stage 1A	T1	N0	M0	Stage 1A	T1	N0	M0
Stage 1B	T2	N0	M0	Stage 1B	T2	N0	M0
Stage 2A	T3	N0	M0	Stage 2A	T3	N0	M0
Stage 2B	T1, T2, T3	N1	M0	Stage 2B	T1, T2, T3	N1	M0
Stage 3	T4	Any N	M0	Stage 3	T1, T2, T3	N2	M0
Stage 4	Any T	Any N	M1		T4	Any N	M0
				Stage 4	Any T	Any N	M1

Tumor extension beyond the pancreas (pT3 in TNM 7) [2, 3] has been reported in up to 90–95% of pancreatic cancer resection specimens [52, 53] leading some to suggest that tumor size should become the defining parameter for pT3 tumors. AJCC/UICC TNM 8 has subsequently accepted size criteria for pT staging of pancreatic cancer (Table 1) [54, 55]. T4 pancreatic cancers are locally advanced (involving the celiac axis or superior mesenteric artery) and in the UK and many other countries are considered to be unresectable. Invasion of the portal vein and/or superior mesenteric vein does not influence T staging.

The introduction of entirely size-based criteria for T staging in TNM 8 will mean that tumor involvement of peripancreatic tissue and/or the intrapancreatic bile duct no longer influences pT staging. However, since pT stage will depend entirely upon the size of the tumor, macroscopic assessment, sampling, and microscopic confirmation will become even more important for the pathologist. It may also be extremely difficult to assess tumor size after neoadjuvant therapy (see Sect. 8).

Table 2 Different AJCC/UICC TNM and R classification of the same pancreatic cancer by different pathologists [2, 3, 44, 46]

Tumor is 2.2 cm diameter, limited to the pancreas, invades the intrapancreatic bile duct, directly invades a single peripancreatic lymph node, and is <1 mm from the superior mesenteric artery margin. How would you stage this PDAC?					
TNM 7			**TNM 8**		
Pathologist A	**Pathologist B**	**Pathologist C**	**Pathologist A**	**Pathologist B**	**Pathologist C**
pT3	pT3	pT2	pT2	pT2	pT2
pN0	pN1	pN1	pN0	pN1	pN1
R0	R1	R0	R0	R1	R1
Stage 2A	Stage 2B	Stage 2B	Stage 1B	Stage 2B	Stage2B
AJCC/UICC TNM 7 states that direct extension into a lymph node is pN1 and that the tumor has to be at the resection margin to be R1			AJCC and UICC TNM 8 remove the ambiguity over T classification and state that direct extension into a lymph node is pN1, but AJCC TNM 8 now considers <1 mm to be a positive (R1) margin		

The accuracy of pN staging depends upon the lymph node yield. Inadequate lymph node sampling can lead to understaging [56, 57] and can also influence the lymph node ratio, which is considered by many to be a more powerful prognostic marker than the overall nodal status [58–60]. However, the number of lymph nodes that should be found (and assessed) in a resection specimen is not universally agreed upon [58, 61–63]. AJCC TNM 7 and 8 state that "optimal histological examination of a pancreatoduodenectomy specimen should include analysis of a minimum of 12 lymph nodes" to accurately stage N0 tumors [2, 44]. UICC TNM 7, however, requires a minimum of 10 lymph nodes [3], but this has been increased to 12 lymph nodes in UICC TNM 8 [46], bringing it into line with the AJCC TNM. Australian [26] guidelines have adopted a minimum of 12 lymph nodes, while the Royal College of Pathologists, UK [36], has proposed that a minimum of 15 lymph nodes should be examined.

While all pathologists agree that a discrete tumor deposit within a lymph node that is not contiguous with the main tumor mass should be considered pN1, there is disagreement whether or not direct invasion of a lymph node by the primary tumor should be considered pN1 (Table 2). Direct lymph node invasion, in the absence of a noncontiguous nodal metastasis, occurs in 9–20% of pancreatic resections. Some authors have suggested that direct invasion does not represent a lymph node metastasis and is equivalent to pN0 prognostically [64]. Others have shown that direct lymph node invasion is associated with an outcome equivalent to that of true (i.e., via lymphatic spread) pN1 [65, 66]. Direct extension of primary tumor into lymph nodes is considered pN1 by AJCC and UICC TNM, as well as by existing national guidelines (e.g., Australia, RCPath, UK) [26, 36].

There is controversy over whether extracapsular lymph node spread in pancreatic cancer is a prognostic factor [65, 67]. A very recent meta-analysis suggests that extracapsular spread is common and associated with a poorer prognosis in pancreatic

ductal adenocarcinoma [68]. However, as the authors acknowledge, there is no standard definition of extracapsular lymph node spread, and pathologists will need to sample lymph nodes with all their surrounding fat to enable such assessment, a practice that is not routinely performed by all pathologists.

The clinical significance of lymph node micrometastasis is controversial, particularly because of the different definitions for micrometastasis. The UICC TNM classification introduced the concept of "isolated tumor cells", defined as single tumor cells or small cell clusters that measure $\leq$0.2 mm in greatest dimensions and can be detected on routine H&E staining or by immunohistochemistry [3]. UICC TNM classifies lymph nodes containing isolated tumor cells as negative (pN0), but adds a suffix (i+) to indicate their presence, i.e., pN0(i+). However, many pathologists would consider such lymph node micrometastases as pN1 [53].

The total number of involved lymph nodes significantly influences survival [66]. Two very recent studies have shown that the number of positive lymph nodes is superior to the lymph node ratio in predicting survival in N1 cases and can distinguish N categories that improve prognostic accuracy [69, 70]. Although AJCC and UICC acknowledge in their general rules for TNM staging that N1, N2, and N3 can be used for "increasing number or extent of regional lymph node involvement," the current AJCC/UICC TNM 7 only uses pN0 and pN1 categories for pancreas cancer [2, 3]. UICC TNM 5 (1997) [51] did separate pN1 for pancreas cancer into pN1a (metastasis in a single regional lymph node) and pN1b (metastasis in multiple regional lymph nodes) based, in part, on the work of Hermanek [71]. Subsequently, most authors did not find significant survival differences between these groups, and both UICC TNM 6 (2002) [72] and 7 (2009) [3] have only included pN0 and pN1. The very recent studies of Strobel et al. [69] and Basturk et al. [70] have shown, with examining high numbers of lymph nodes, that the total number of positive lymph nodes is a strong prognostic predictor. AJCC and UICC TNM 8 have now introduced lymph node-positive categories based on the number of positive lymph nodes using the cutoffs of 0 (pN0) versus 1 to 3 (pN1) versus 4 or more (pN2) (Table 1) [44, 46].

Neoadjuvant Therapy

Neoadjuvant therapy is increasingly used in the treatment of patients with potentially resectable pancreatic cancer, especially patients with borderline-resectable disease, and the pathologist provides key outcome parameters in assessing the degree of tumor regression and completeness of excision in the resection specimen.

Following a response to neoadjuvant therapy, there will be a reduction in the number of tumor cells with areas of the tumor replaced by fibrosis. Macroscopic distinction between the tumor, fibrotic areas of tumor regression, and background fibrosis of (chronic or obstructive) pancreatitis can be extremely difficult, if not impossible (Fig. 6) [73]. PDAC has a highly infiltrative growth pattern, and residual tumor may still be present within macroscopically nonneoplastic tissue following neoadjuvant therapy [74]. Therefore, extensive sampling is required to assess the extent and size of the residual tumor and its relationship to the margins. Sampling the

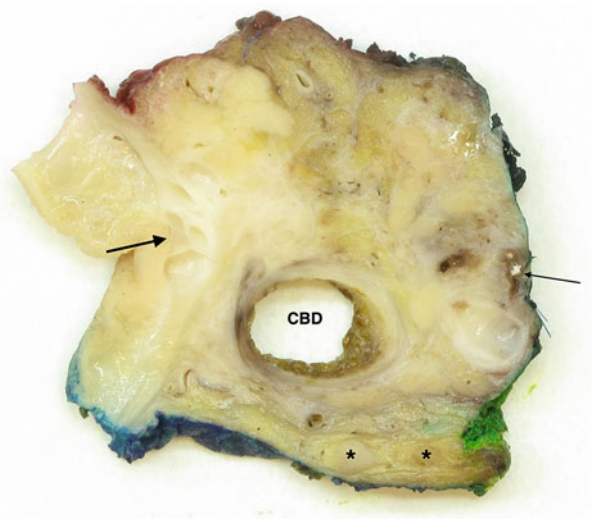

Fig. 6 Axial specimen slice following neoadjuvant therapy. Ill-defined areas of abnormal, fibrous tissue have replaced large parts of the pancreatic parenchyma and the duodenal wall. There are foci of cyst formation (*thick arrow*) and necrosis (*thin arrow*). Note the dilated common bile duct (*CBD*) as a result of metal stenting

entire resected pancreas is recommended to confirm a complete response [74]. Tumor necrosis may be a marker of therapy effect, but it also occurs in untreated cancers and, therefore, cannot be used reliably as a marker of tumor regression [75]. Following neoadjuvant therapy and a good tumor response, the pathologist is unlikely to be able to determine the primary origin of the cancer, and, therefore, bile duct cancers may be included in, and influence, studies of neoadjuvant therapy.

There are several schemes for histologic grading of tumor regression in use, based either on assessment of the amount of tumor destruction or on the amount of residual tumor [32, 75–79]. The prognostic significance of these tumor regression grading systems in post-therapy pancreatectomy specimens is largely unknown. A recent study found that patients with a complete response (Table 3) (Evans grade 4, CAP grade 0) or minimal residual disease (Evans grade 3, CAP grade 1) had better disease-free survival and overall survival than patients with moderate or no response. There was no difference in disease-free survival or overall survival between the CAP grades 2 and 3 [80]. This led the authors to propose a modified CAP grading system (Table 3) [80]. They also found that tumor regression grade is an independent prognostic factor for survival in multivariate analysis.

There are difficulties with these tumor regression grading systems, including reproducibility. Detection of residual tumor cells is straightforward, but there are no morphological features that will clearly delineate viable from nonviable tumor cells. Cytopathic effects can be seen in tumor cells following neoadjuvant therapy, but they can also be detected in untreated tumors that have been affected by inherent tumor-related ischemia [75]. The main difficulty is the assumption that the pathologist can assess the original (pre-therapy) size of the tumor and the extent of the fibrosis that is treatment-induced [74]. Extensive sampling of the resection specimen is essential for this, but distinguishing tumor-related fibrosis from neoadjuvant therapy-induced fibrosis microscopically can be just as difficult as it is macroscopically. Rates of complete

Table 3 Tumor regression grading systems for PDAC

The tumor regression grading system of Evans et al. [77]	
Grade	Extent of tumor cell destruction/residual tumor
I	Little (<10%) or no tumor destruction
2a	Destruction of 10–50% of tumor cells
2b	Destruction of 51–90% of tumor cells
3/ 3M[a]	Few (<10%) viable-appearing tumor cells
4/ 4M[a]	No viable tumor cells
The tumor regression grading system of the College of American Pathologists [32]	
Grade	Proportion of residual viable tumor
0	No viable cancer cells (complete histologic response)
1	Single cells or rare small groups of cancer cells (near complete response)
2	Residual cancer with evident tumor regression, but more than single cells or rare small groups of cancer cells (partial response)
3	Extensive residual cancer with no evident tumor regression (poor or no response)

The tumor regression grading system of Chatterjee et al. [80]	
Grade	Proportion of residual viable tumor
0	No residual cancer
1	Minimal residual cancer (single cells or small groups of cancer cells, <5% residual cancer)
2	5% or more residual cancer

[a]Addition of the M suffix indicates abundant residual mucin pools

tumor regression vary considerably in the literature, with rates ranging from <3% to >30% [74, 80, 81]. These different results are clearly influenced by the extent of tissue sampling from the resection specimen, making it difficult to compare the efficacy of different neoadjuvant therapy regimens in different studies [74].

There is currently controversy over the appropriate minimum clearance to define microscopic margin involvement (R1) in "treatment-naïve" pancreatic cancer specimens (see Sect. 5). With neoadjuvant therapy-induced destruction of tumor cells, the distances between the remaining tumor cells increase, and a minimum clearance of 1 mm cannot guarantee the absence of residual tumor beyond the resection margin [30, 74]. The appropriate minimum clearance following neoadjuvant therapy is unknown, but a distance of 5 mm has been proposed recently [82]. Reported R1 rates post neoadjuvant therapy vary from 0% to 100%, reflecting differences in margin evaluation and definitions of R1 [74].

For tumor staging following neoadjuvant therapy, only the presence of tumor cells in the resection specimen is used to determine the stage. In the current AJCC/UICC TNM 7 [2, 3], many of these post neoadjuvant therapy resection specimens are still staged as pT3 because foci of residual tumor are commonly found in the peripancreatic tissue. Implementation of TNM 8 [44, 46], where T stage for pancreatic cancer is entirely related to tumor size, will pose challenges for the pathologist in the post neoadjuvant therapy resection specimen. When there is only one focus of

residual tumor, the size of this focus can be measured to determine the ypT stage. If, however, there are multiple scattered residual tumor foci in the resection specimen, then is the size (and ypT stage) determined by the size of the largest tumor focus, the sum of the sizes of all of the foci, or the maximum dimension of the area containing residual tumor?

Conclusion

Despite much progress in the diagnosis and treatment of pancreatic cancer, there is still a lack of consensus on the assessment and classification of basic tumor characteristics such as tumor origin, tumor stage, and resection margin involvement. In 2012, Rau et al. [7] highlighted the requirement for an internationally accepted and standardized, but technically and financially feasible, pathology reporting of pancreatic cancer resection specimens. Five years on, there has been some progress, particularly with the updating of national pathology guidelines [26, 32, 36, 37] and the use of pathology reporting proformas. Use of the latter is recognized to facilitate accurate and complete pathology reports [83], but the content of these proformas still differs between the published guidelines.

There are still different protocols in use for handling and sampling pancreas cancer resection specimens, including after neoadjuvant therapy. The International Study Group for Pancreatic Surgery (ISGPS) has endorsed the use of the axial slicing technique [27], but this method is not universally accepted by pathologists. There are differing opinions over what constitutes a resection margin, when the margin should be considered involved, and how many lymph nodes should be examined. Differences in interpretation of T staging of pancreatic cancer using the current AJCC/UICC TNM 7 [2, 3] have largely been overcome by the change to T staging in AJCC/UICC TNM 8 [44, 46]. These rather dramatic changes in T staging criteria reflect the ongoing research and discussion on this important issue but, in turn, introduce new difficulties. TNM 8 is not due for implementation until January 2018. It remains to be seen whether these recent changes to TNM will correlate better with patient outcome. There still remains a need for international consensus on many aspects of pancreatic cancer pathology reporting.

Cross-References

- ▶ MRI and MRCP for Diagnosis and Staging of Pancreatic Cancer
- ▶ Neoadjuvant Chemotherapy in Pancreatic Cancer
- ▶ New Japanese Classification of Pancreatic Cancer
- ▶ Pancreatic Adenocarcinoma: CT and PET/CT
- ▶ Pathologic Classification and Biological Behavior of Pancreatic Neoplasia
- ▶ Staging and Postoperative Outcomes Using the International Study Group of Pancreatic Surgery (ISGPS) Classifications
- ▶ Venous Resection in Pancreatic Cancer Surgery

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Staging and Postoperative Outcomes Using the International Study Group of Pancreatic Surgery (ISGPS) Classifications

T. Welsch and J. Weitz

Contents

Abstract

The International Study Group of Pancreatic Surgery (ISGPS) established consensus definitions of postoperative pancreatic fistula (POPF), postpancreatectomy hemorrhage (PPH), and delayed gastric emptying (DGE) and thereby covered the major specific complications of pancreatic surgery. A threefold increase of the amylase content in abdominal wound drains compared with serum level on or after the third postoperative day defines POPF; early and late (>24 h) PPH is defined by mild and moderate bleeding according to the drop of hemoglobin or the need for transfusion requirement, and the inability to tolerate solid oral intake after the first postoperative week defines DGE. All three consensus definitions are classified into three grades: A, B, and C. These grades stratify the clinical effect (the illness of the patient and the need for intervention), associated mortality, hospital stay, and economic costs. All definitions have been validated using large cohorts of patients and show different outcome data at different centers, even

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when high-volume centers are compared with each other. A minor degree of equivocacy of the original definitions is also acknowledged. The clinically relevant grades are most important, and the prevalence of grade B/C complications based on the ISGPS definitions occurs at the following median rates: 12–20% (POPF), 2–11% (PPH), and 6–17% (DGE).

Keywords
Pancreatic surgery · Postoperative pancreatic fistula · Postpancreatectomy hemorrhage · Delayed gastric emptying · Definition

Introduction

There is no doubt that pancreatic surgery carries a relatively high risk of postoperative morbidity. Even at high-volume centers, the overall morbidity is reported at 42–56% and is substantially increased if pancreatic surgery is performed as multivisceral or extended resection [1, 2]. The most critical surgical complications are postoperative pancreatic fistula (POPF), postpancreatectomy hemorrhage (PPH), and delayed gastric emptying (DGE), which may all be related to the surgical technique but likewise may be interrelated with each other. The latter two frequently occur as a sequela of an established POPF. Because the surgical technique, surgical experience, and patient selection can significantly modulate the prevalence of the previously mentioned surgical complications, these complications have had and are still exerting an immense effect on surgical research. However, effective research and transparent benchmarking necessitates uniform and unequivocal definitions of the respective complications, i.e., the widespread use of the same "currency." A literature search in the year 2004 identified 26 different definitions of POPF resulting in a significant variation of the calculated prevalence of POPF when applied to a training set of patients [3]. Likewise, the terminology used in different studies was diverse: pancreatic leak, insufficiency, fistula, or postoperative bleeding, hemorrhage, or erosion bleed, to name only a few. In 2005, the International Study Group of Pancreatic Surgery (ISGPS) sought to put an end to the diversity and confusion of postpancreatectomy complications when publishing the consensus definition for POPF [4]. The final consensus definition was simple and easy to assess and use, and the severity based on the clinical effect was weighted into three grades: A, B, and C. Two years later, the ISGPS consensus definitions of PPH and DGE were published, and a grading A–C was introduced accordingly. Since then, the three ISGPS definitions have changed pancreatic surgery reporting and research tremendously. The present chapter underlines that the ISGPS consensus definitions have become a pivotal pillar of pancreatic surgery and enable differentiation of patient illness, hospital stay, or costs. It also becomes clear that the ISGPS definitions may require minor revision for further improvement of their use.

Postoperative Pancreatic Fistula

The ISGPS definition has been well accepted and adopted for the staging of patients with POPF. The original consensus definition publication in 2005 [4] has been cited 1,762 times (until June 9, 2016), with an average number of citations per year of 147 (Fig. 1), making it one of the most cited articles in surgical research. According to the original publication, POPF is defined as "drain output of any measurable volume of fluid on or after postoperative day 3 with an amylase content greater than three times the serum amylase activity" [4] (Table 1). Three different grades, A, B and C, delineate the effect on the patients' clinical course and are summarized in Table 1. Briefly, grade A fistulas are asymptomatic and require no change in clinical management; grade B fistulas prompt diagnostic, medical, or interventional adjustment of the standard management (e.g., antibiotics, somatostatin analogs, computed tomography [CT] scan, drain replacement), whereas patients with grade C fistulas are generally critically ill and require a major change in management and potentially a surgical reoperation plus intensive care. However, the original definition is equivocal with respect to the discrimination of grade B/C fistulas. Although the text reads that a postoperatively placed CT-guided drainage of an intra-abdominal fluid

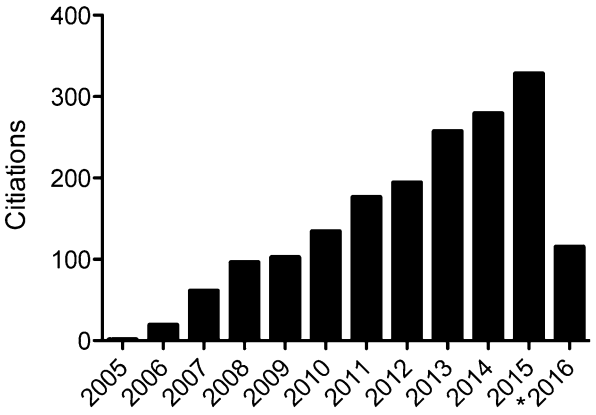

Fig. 1 Annual citations of the consensus definition of POPF since its original publication in 2005. The data were obtained from the Web of Science database. In total, there were 1,762 citations and an average number of citations of 146.8 per year. *As of June 2016

Table 1 ISGPS definition of POPF (Modified from Bassi et al. [4])

Definition		Drain output of any measurable volume of fluid on or after postoperative day 3 with an amylase content greater than three times the serum amylase activity		
Grade		Clinical condition/US/CT finding	Specific treatment/ intervention	Hospital stay
	A	Well/negative	No	Not prolonged
	B	Often well/negative or positive	Usually yes/drainage	Usually prolonged
	C	Ill appearing/positive	Yes/reoperation	Prolonged

CT computed tomography, *US* ultrasound

Table 2 ISGPS definition of PPH (Adapted from Wente et al. [17])

Grade	Time of onset and severity[a]		Clinical condition	Therapeutic consequence
	Early (≤24 h)	Late (>24 h)		
A	Mild		Well	No
B	Severe	Mild	Often well/ intermediate/very rarely life-threatening	Transfusion of fluid/blood, intensive care unit (or ICU), therapeutic endoscopy, embolization, relaparotomy for early PPH
C		Severe	Severely impaired, life-threatening	Localization of bleeding, angiography, and embolization (endoscopy) *or* relaparotomy, ICU

	Mild	Severe
Blood loss	Decrease in hemoglobin concentration <3 g/dl	Decrease in hemoglobin concentration by ≥3 g/dl
Volume resuscitation/blood transfusions	Volume resuscitation or blood transfusions (2–3 units of packed cells within 24 h of the end of operation or 1–3 units if later than 24 h after operation)	Clinically significant impairment (e.g., tachycardia, hypotension, oliguria, hypovolemic shock), need for blood transfusion (>3 units of packed cells)
Need for invasive (interventional or operative) treatment	No	Yes

[a]Severity of PPH

collection characterizes a grade C fistula, Table 2 of the original publication indicates that grade C fistulas require surgical re-exploration [4]. Consequently, some studies categorized patients with postoperative, interventional CT drainage but without re-exploration as grade B and others as grade C, leading to different outcomes. A recent retrospective analysis of 2,955 patients after pancreatic surgery at a high-volume center with a total POPF rate of 13.6% (grades A–C) proposed that cases with interventional drainage, but without re-exploration, should be assigned to grade B, and cases with surgical re-exploration be assigned to grade C only. The rationale was a significant increase in the hospital stay and POPF-associated mortality (37%) if a reoperation was performed [5]. Therefore, a future revision of the consensus definition might consider these data to minimize equivocacy.

Regardless, previous studies have demonstrated that the different grades A–C of the ISGPF definition discriminate well among the clinical condition of the patient, the need for a change in management, the associated hospital stay duration, and the economic burden [6–8]. The hospital stay duration was 8, 13, and 35 days in a US medical center and 11–15, 22–24, and 39–46 days in European centers in grade A, B, and C fistula cases, respectively. The respective associated total hospital costs were calculated to be approximately $18,100/$25,200/$119,100 and €11,700/€25,700/€59,500 for grades A, B, and C, respectively [6–8].

Before the introduction of the consensus definition, the prevalence of POPF varied significantly among different centers and was reported between 2% and 20%, and even higher [4]. The uniform use of the ISGPS definition enables meta-analysis of various studies to determine the actual POPF rate for benchmarking. One such meta-analysis was published in 2014 and included articles until the year 2011 [1]. By the year 2011, over 70% of the publications reporting on POPF had used the ISGPS consensus definition. In total, the meta-analysis identified more than 50 studies that used the ISGPS POPF definition. The analyzed studies included more than 13,000 patients after pancreatic resections. The median POPF prevalence (grades A–C) of these studies was 21.9% in retrospective and 28.6% in prospective studies [1]. Interestingly, the prevalence of symptomatic and clinically relevant grade B/C fistulas was almost equal in both study groups (12%). If a subgroup analysis was performed according to the type of surgical resection (pancreatoduodenectomy versus distal pancreatectomy), there was still a lower overall POPF prevalence in retrospective studies in the two subgroups, and prospective studies reported POPF at 26.1% (pancreatoduodenectomy) and 36.1% (distal pancreatectomy). The latter data originate from the DISPACT trial, analyzing the closure technique of the pancreatic remnant after distal pancreatectomy in a randomized, controlled design, in which the prevalence of grade B/C POPF alone was 20.5% [9].

A more recent German multicenter, randomized, controlled trial including only pancreatic head resections at academic high-volume centers (i.e., the RECOPANC trial) compared pancreaticogastrostomy with pancreatojejunostomy, and the primary outcome of the trial was the prevalence of clinically relevant grade B/C POPF [10]. Interestingly, grade B/C POPF was observed in 21% of the patients, and there was no significant difference between the two surgical techniques. The data from the two multicenter trials DISPACT and RECOPANC indicate that the rate of grade B/C fistulas is approximately 20% and had most likely been underreported in previous trials (e.g., 12%). Reasons for this discrepancy could be a less accurate monitoring in prospective or irregular data collection in retrospective trials.

The ISGPS definition of POPF also contributes to improving the data quality of studies investigating the prophylactic use of somatostatin analogs for the prevention of POPF. This controversial topic still lacks solid data because only a few of the many studies on prophylactic somatostatin analogs have applied the ISGPS definition, and most studies were in fact conducted before 2005 [8, 11]. There was only one adequately powered, randomized, controlled trial in recent years that considered the ISGPS definition, unfortunately as a secondary outcome [8, 12]. In this unicenter trial, the novel somatostatin analog pasireotide was shown to significantly lower grade B/C POPF compared with placebo treatment (7.9% versus 16.9%, $P = 0.02$) [12]. Future trials will add more evidence to prophylactic somatostatin analog treatment using the ISGPS definition.

In summary, the ISGPS definition of POPF was a major contribution to academic pancreatic surgery and is considered the international standard for data assessment and reporting. Minor modification of the exact definition of the different grades will further improve its discrimination of the clinical and economic effect. Accurate

documentation of the POPF variables (the amylase content of wound drains on or after the third postoperative day, the need for specific treatment or intervention, and the clinical condition of the patient) and exact application of the definition result in approximately 20% of grade B/C fistulas (no specific patient selection) with current best medical practice. If cases with reoperation for POPF will be classified as grade C only, then most of the 20% will be grade B fistulas.

Postpancreatectomy Hemorrhage

The definition of postpancreatectomy hemorrhage (PPH) is more complex compared with the POPF definition and requires a subdivision. It is defined by three parameters: onset ($\leq$24 h [early PPH] or >24 h [late PPH] after the index operation), location (intra- or extraluminal), and severity (mild or severe) [13]. Based on these parameters and the clinical effect, PPH is categorized into the three grades: A, B, and C (Table 2). The PPH definition had been cited 368 times between 2007 and 2016, with an average number of citations of 36.8 per year (Fig. 2). Nevertheless, the meta-analysis and review of the ISGPS consensus definitions by Harnoss et al. found that the acceptance of the PPH definition was lower compared with the POPF and DGE definitions by the year 2011 and was cited by approximately 20% of research articles reporting on PPH issues [1]. The reason for the lower acceptance is not obvious, because the definition logically characterizes and distinguishes the different types of bleeding. In particular, the clinically relevant PPH grades B/C include early severe PPH, which is often caused by technical (surgical) issues, and late severe PPH (grade C), which is generally elicited by an established POPF and erosion hemorrhage of visceral arteries. Mild late PPH was introduced to cover sentinel bleedings. However, if the definition of mild late PPH is strictly followed (drop of hemoglobin <3 g/dl or transfusion of 1–3 units of packed red blood cells [PRBC] during the hospital course >24 h after the index operation), many more (false-positive) patients in addition to the ones with a sentinel bleed are captured by the definition [14]. Therefore, a

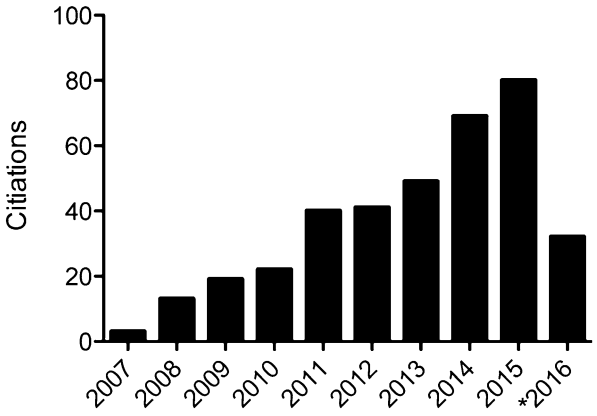

Fig. 2 Annual citations of the consensus definition of PPH since its original publication in 2007. The data were obtained from the Web of Science database. In total, there were 368 citations and an average number of citations of 36.8 per year. *As of June 2016

revision of the definition should be discussed, in which only patients with a novel, small amount of blood loss through the abdominal drains (sentinel bleeding, clinical definition) – regardless of any drop of hemoglobin or a need of transfusion requirement – are considered for the mild late PPH subgroup.

Validation studies of the PPH definition using large high-volume cohorts have demonstrated that the three PPH grades discriminate the cases well among the need for transfusion requirements, intensive care unit stay, hospital stay, and mortality [14, 15]. The prevalence of grade C POPF in the validation studies was 9.2 and 4%, and the associated mortality within this subgroup was 16.4 and >25%, respectively. The review by Harnoss et al. pointed out the difference among the retrospective (7.1%), prospective (2.2%), and validation (24.4%) use of the PPH definition for grades B and C, which prompted further discussion. Although a retrospective assessment of all PPH grades on the basis of databases can miss late mild cases (sentinel bleeding), 2.2% of clinically relevant PPH in prospective studies can be considered a very low benchmark that is not achieved in general – not even by all high-volume centers.

Importantly, the allocation to grade B/C PPH is independent of the management (operative or interventional management) of the hemorrhage, and good data exist that show no significant change in the prevalence of PPH over the last two decades, but a trend toward more interventional management of grade C PPH [16].

In summary, the PPH definition is as important as the POPF definition and discriminates well the clinical effect of the bleeding. The prevalence of clinically relevant grade B/C PPH varies at different centers and patient cohorts and resides between 2% and 11%.

Delayed Gastric Emptying

Delayed gastric emptying (DGE) is a multifactorial complication, and the pathophysiology is still not completely understood. Therefore, the definition of objective and assessable parameters is challenging. The ISGPS defined DGE as the "inability to return to a standard diet by the end of the first postoperative week and includes prolonged nasogastric intubation of the patient" [17]. Analogous to the other ISGPS definitions, the three grades A, B, and C were further defined based on the effect of the clinical course and postoperative management. Briefly, grade A DGE describes patients who are unable to tolerate solid oral intake by the first postoperative week, but require no major change of management or hospital stay. Grade B/C DGE describes the inability to tolerate solid oral intake by the second or third postoperative week and the need for medical or interventional specific treatment, respectively (Table 3). DGE itself is not life-threatening if aspiration is anticipated and avoided. However, grade B/C DGE can frequently mirror intra-abdominal hematoma or fluid collections (e.g., POPF, abscess). Therefore, the ISGPS definition describes patients with grade C DGE in a severe clinical condition and at an increased risk for critical comorbidities.

During the time period from the original publication until the composition of this article (2007–2016), the definition has been cited 562 times, or 56.2 times, per year (Fig. 3). In 2011, the acceptance of the DGE definition was as high as the POPF

Table 3 ISGPS definition of DGE (Adapted from Wente et al. [17])

DGE grade	NGT required	Unable to tolerate solid oral intake by POD	Vomiting/ gastric distension	Use of prokinetics
A	4–7 days or reinsertion > POD 3	7	±	±
B	8–14 days or reinsertion > POD 7	14	+	+
C	>14 days or reinsertion > POD 14	21	+	+

POD postoperative day, *NGT* nasogastric tube

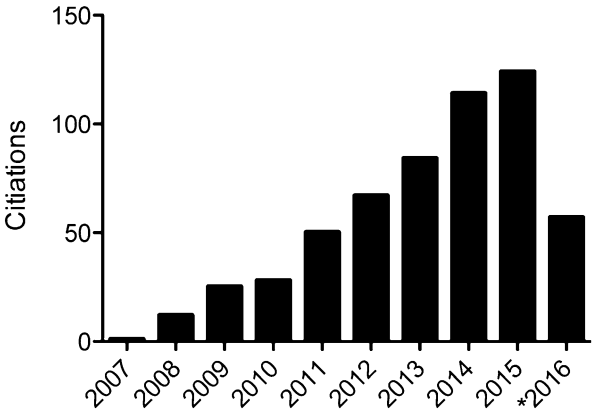

Fig. 3 Annual citations of the consensus definition of DGE since its original publication in 2007. The data were obtained from the Web of Science database. In total, there were 562 citations and an average number of citations of 56.2 per year. *As of June 2016

definition, and approximately 70% of the articles on DGE used the ISGPS definition [1]. However, the ISGPS-based prevalence of DGE was significantly different in prospective (10.8%), retrospective (20.8%), and validation studies (33.3%) [1]. Grade B/C DGE occurred at a median rate between 6 and 17%. The real prevalence of DGE might even be higher. One validation study reported DGE in 44.5% of the cases after pancreatoduodenectomy. The three grades A, B, and C are associated with prolonged total hospital stay and intensive care unit stay [18]. In the United States, the hospital charges increased over $10,000 with each severity grade [19]. It became further evident that the DGE definition is generally feasible and applicable but has limits in patients with prolonged intensive care unit (ICU) stay for other complications (e.g., parenteral nutrition, nasogastric tube placement for endotracheal intubation) [18, 19]. This is the reason why some studies introduced the term "primary DGE" when referring to DGE that is not caused or associated with other obvious complications [19]. A relative high prevalence of DGE was also reported by a recent German multicenter, randomized, controlled trial investigating pancreatojejunostomy and pancreaticogastrostomy (RECOPANC) [10]. In this trial, DGE occurred in 39% of the patients.

The physiology of "primary DGE" is incompletely understood, but data exist to show that surgical technique can affect the prevalence of the ISGPS DGE. Although there are controversial results, the antecolic reconstruction of the gastrojejunostomy appears to lower DGE after classic pancreatoduodenectomy [20, 21]. Some centers have examined the technique of pylorus ring resection over and found a significant reduction in the rate of DGE [22–24]. Furthermore, a recent meta-analysis disclosed that minimally invasive pancreatoduodenectomy also lowered the incidence of DGE [25].

Conclusions

All three ISGPS definitions are logical and feasible and can be considered standard for assessing and reporting POPF, PPH, or DGE. The definitions have been successfully validated, and the different grades discriminate well the clinical effect of the respective complication, which is generally associated with a stepwise increase in hospital stay, intensive care unit stay, mortality rate, and economic costs. There are still discrepant prevalence outcomes of POPF, PPH, and DGE when the ISGPS definitions are applied. These differences can be partly explained by interpretation of the definition or inaccurate data monitoring but also reflect different outcomes in various hospitals and centers. This was the primary aim of the ISGPS definitions: to enable benchmarking of the performance, transparency, and ways for improvement. Each of the three definitions has some minor limitations in its original form and would benefit from some minor modifications.

Cross-References

- ▶ Arterial Resection in Pancreatic Cancer
- ▶ Interventional Radiology for Pancreatic Cancer
- ▶ Staging and Postoperative Outcomes using the International Study Group of Pancreatic Surgery (ISGPS) Classifications
- ▶ Surgical Resection for Pancreatic Cancer using the International Study Group of Pancreatic Surgery (ISGPS) Classifications

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Borderline Resectable Pancreatic Cancer

Gauri R. Varadhachary

Contents

Abstract

Rigorous criteria are essential to define resectability of PDAC, which allows for accurate pretreatment staging and planning stage-specific therapy. Tumors of borderline resectability have emerged as a distinct subset, and these patients are at a high risk for margin positive resection. The *intergroup* criteria for BRPC includes: (1) an interface between the tumor and SMV–PV $\geq 180^{\circ}$ of the vein circumference; (2) short-segment occlusion of the SMV–PV with normal vein above and below the obstruction amenable to resection and reconstruction; (3) short-segment interface of any degree between tumor and HA with normal

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artery proximal and distal to the interface amenable to arterial resection and reconstruction; and (4) interface between the tumor and SMA and/or CA measuring <180° of the circumference of the artery. Two multicytotoxic regimens approved for metastatic disease, 5-flurouracil with oxaliplatin and irinotecan (FOLFIRINOX) and gemcitabine with *nab*-paclitaxel (Gem-*nab*P), are incorporated in the preoperative management of BRPC in many centers although high-level evidence data on these regimens in the neoadjuvant setting are not yet available. Those with radiographic stability or regression and an improvement in serum tumor markers (CA19-9) may proceed to pancreatectomy and may require vascular resection and reconstruction. Prospective clinical trials with well-defined eligibility will help determine the treatment strategies. Additionally, prognostic and predictive biomarkers are urgently needed in therapy planning.

Keywords
Pancreatic adenocarcinoma · preoperative chemotherapy · neoadjuvant · borderline resectable · biomarkers

Background

Pancreatic ductal adenocarcinoma (PDAC) is a systemic disease in most patients; two thirds of patients have locally advanced or metastatic disease at the time of diagnosis. Twenty to 25% of patients present with a potentially resectable or borderline resectable pancreatic adenocarcinoma (BRPC). Although over the last two decades, there has been a small improvement in the overall 5-year survival rate of patients undergoing pancreatectomy, there is no seismic shift in the disease free interval and median overall survival of patients undergoing potentially curative resection [1–2]. Beside the concern for micro metastatic disease at the time of attempted curative resection, numerous studies have reported on poor outcomes for patients who undergo an incomplete margin positive resection, with survival similar to patients with locally advanced pancreatic cancer [3–4].

Determining resectability of the primary pancreas tumor is essential to the initial staging evaluation. This is best accomplished by a computerized tomography (CT) scan optimized for pancreatic imaging [5]. Based on this high quality CT imaging, in the past, pancreatic tumors have typically been classified as resectable, locally advanced, or metastatic. In the era of the multidetector CT optimized for pancreatic imaging, tumors of "borderline resectability" have emerged as a distinct subset of PDAC [6–10]. This distinction between resectable and BRPC is crucial to plan appropriate management algorithms that impact patient' quality of life, clinical trial designs, and eventual survival. The attempt to standardize the definition of BRPC is work in progress and has made strides in the last decade. The criteria have been modified over time, beginning with descriptions from M. D. Anderson Cancer Center (MDACC), followed by National Comprehensive Cancer Network (NCCN), and consensus conferences, the first being sponsored by the

AHPBA/SSAT/SSO and lastly with the intergroup definition which is used in currently planned prospective trials in the United States [11]. Additionally, in the last several years, two multicytotoxic regimens are approved for the management of metastatic disease, 5-flurouracil with oxaliplatin and irinotecan (FOLFIRINOX) and gemcitabine with *nab*-paclitaxel (Gem-*nab*P), and these have been incorporated in the preoperative management of BRPC with a neoadjuvant intention [12–13]. Here in, the author reviews the working definition of BRPC, including the anatomic and patient-related factors that constitute borderline resectable tumors and provide a framework for management of patients with tumors of borderline resectability. Given the lack of adequate multi-institutional prospective data, and the intrinsic heterogeneity of the disease entity, there is ongoing debate with respect to upfront resection in select patients vs. sequencing and duration of neoadjuvant therapies, standardization of surgical techniques, patient selection, and role of novel agents and biomarkers.

Preoperative Staging for Resectable and BRPC and the Role of the Multidetector CT Scan

Several modalities have been employed for the preoperative staging of pancreatic cancer including multidetector computerized tomography (MDCT), endoscopic ultrasound (EUS), endoscopic retrograde cholangiopancreatography (ERCP), magnetic resonance imaging (MRI), magnetic retrograde cholangiopancreatography (MRCP), and serum tumor markers [14–17]. There is a consensus that MDCT optimized for pancreatic imaging is the best modality to determine local tumor resectability [18]. The main limitation of this technique is its low sensitivity for low-volume hepatic or peritoneal metastases. Studies suggest that in approximately 20% of the patients who have a potentially resectable or BRPC on MDCT preoperatively, CT occult metastatic disease is found on exploration [19–20], and therefore selective application of laparoscopy and laparoscopic ultrasound is commonly performed in high volume cancer centers. It is beneficial, whenever possible, to perform a CT scan prior to biliary decompression procedures since postprocedure pancreatitis, if it occurs, may obliterate the vascular planes and preclude accurate assessment of the extent of disease. The MDCT post-processing techniques such as maximum intensity projection (MIP) images and volume rendering can help identify important vascular variants. In patients in whom CT scan suggests an isodense mass in the pancreatic head, EUS with EUS fine needle aspiration (EUS-FNA) and biopsy can confirm the diagnosis without a significant risk of acute pancreatitis and also define the relationship of the tumor to the surrounding vasculature. Raut and colleagues reported retrospective data on EUS-FNA in 233 patients who presented with CT evidence of a pancreatic mass or a malignant biliary stricture [21]. In this series, a diagnosis of cancer was established in 216 patients (93%); 15 patients (6%) were found to have benign disease, and the final diagnosis remained unknown in two patients (1%). The sensitivity, specificity, and accuracy of EUS-FNA for diagnosis of a pancreatic malignancy were 91%, 100%, and 92%, respectively.

BorderlineResectable Pancreatic Cancer: Definitions and CT Based Criteria

The purpose to establish objective radiographic criteria is to allow decisions regarding tumor resection to be made by a multidisciplinary group working jointly rather than it being purely a surgeon's prerogative with a decision made at the time of laparotomy. The definitions have evolved to remove inexact terms like "impingement", "abutment," and "involved." These definitions are very important for standardization, and therefore the conduct of clinical trials involving the use of preoperative or adjuvant therapies.

NCCN describes borderline resectable pancreatic head (and body) cancer as tumor abutment on SMA, severe unilateral SMV or PV impingement, gastroduodenal artery (GDA) encasement up to its origin from the hepatic artery, or colon and mesocolon invasion [10]. At MDACC, patients with borderline resectable pancreatic cancer include those whose tumors exhibit: short-segment encasement of the hepatic artery which is amenable to resection and reconstruction without evidence of tumor extension to the celiac axis; abutment of the SMA to involve $\leq 180^\circ$ of the circumference of the artery; or short-segment occlusion of the SMV, PV, or SMPV confluence with a suitable option for vascular reconstruction due to a normal SMV below and PV above the area of tumor involvement. The AHPBA, SSO, and SSAT Consensus Conference (2008) definition includes tumor associated deformity of the SMV–PV, abutment of the SMV–PV $\geq 180^\circ$, short-segment occlusion of the SMV–PV amenable to resection and reconstruction, short-segment involvement of the hepatic artery (HA) or its branches amenable to resection and reconstruction, and abutment of the SMA ($<180^\circ$). Most recently, several cooperative groups, including the Southwest Oncology Group, (SWOG) Eastern Cooperative Oncology Group (ECOG), and Radiation Therapy Oncology Group (RTOG), proposed a definition referred to as the *intergroup definition*, and it was used for the completed Alliance pilot trial (A021101) using preoperative modified-FOLFIRINOX followed by capecitabine based, 5040 Gy external beam radiation therapy prior to intended surgery. The intergroup criteria consists of the following: (1) an interface between the tumor and SMV–PV $\geq 180^\circ$ of the vein circumference; (2) short-segment occlusion of the SMV–PV with normal vein above and below the obstruction amenable to resection and reconstruction; (3) short-segment interface of any degree between tumor and HA with normal artery proximal and distal to the interface amenable to arterial resection and reconstruction; and (4) interface between the tumor and SMA and/or CA measuring $<180^\circ$ of the circumference of the artery (11).

Anatomic CT Based Criteria for Borderline Resectable Pancreatic Cancer: (Defined as MDACC, Type A)

Interface Between the Tumor and SMA/CA Measuring $<180^\circ$ of the Circumference of the Artery

Multiple groups have defined the tumor vessel orientation in PDAC over the last 20 years. The older classification, reported by Loyer and colleagues, categorizes the

extent of tumor abutment of the vessel wall from type A to type F [22]. In type A, there is a fat plane around the vessel; in type B, normal pancreatic parenchyma separates the tumor from the vessel; in type C, the tumor is inseparable from the vessel but the points of contact form a convexity against the vessel; in type D, partial encircling of the vessel is present and the contact point forms a concavity against the vessel wall; in type E, the tumor is completely encasing the vessel; and in type F, the tumor is completely occluding the vessel. This classification system describes tumor-vessel relationships and does not differentiate between venous and arterial involvement.

Lu and colleagues reported an alternate grading system where tumor involvement of the PV and SMV and the celiac axis, hepatic artery, and SMA is graded on a 0–4 scale based on circumferential contiguity of tumor to vessel [23]. Based on this grading system, no tumor contiguity to a vessel denotes grade 0. In grade 1, tumor is contiguous to less than one-quarter circumference of the vessel; in grade 2 the tumor is contiguous between one-quarter and one-half of the circumference; in grade 3, between one-half and three-quarters circumference; and in grade 4, the tumor contiguity is greater than three-quarters circumference of the vessel or there is vessel constriction. Using this system, they evaluated 25 patients with pancreatic adenocarcinoma who underwent preoperative pancreatic-phase thin-section helical CT followed by pancreaticoduodenectomy. Surgical results were then correlated with the CT grading system and evaluation was possible for 80 vessels. All vessels graded 0 (48 vessels) or 1 (3 vessels) were resectable, and most of those graded 3 (7/8 vessels) and all of those graded 4 (14/14 vessels) were unresectable. A threshold between grades 2 and 3, which corresponded to tumor involvement of one-half circumference of the vessel, yielded the lowest number of false-negatives and an acceptable number of false-positives for unresectability. The authors concluded that such a threshold would have yielded a sensitivity of 84%, a specificity of 98%, a positive predictive value of 95%, and a negative predictive value of 93% for unresectability of the cancer based on the vessels studied. They concluded that if tumor involves more than one-half the circumference of the vessel, it is highly specific for unresectable tumor. However, the authors did not have a standardized approach to vascular resection and there was no reported pathologic data on resection margin status (R0 vs. R1/2), making it difficult to interpret these results. In another study by Saldinger and colleagues, helical CT and CT angiography with three-dimensional reconstruction prospectively staged a total of 100 patients with periampullary neoplasms [24]. Vascular involvement was graded from 0 to 4, with grade 0 representing no vascular involvement and grade 4 representing total encasement of either the SMA or SMV. Resectability rates for grades 0, 1, 2, and 3 were 96%, 100%, 50%, and 9%, respectively, for an overall resectability rate of 76%. Valls and colleagues have reported on the presence of "reticular opacities," which are small strands arising from the tumor and abutting the vessels in some of their patients [25]. This appearance may have more significance in the setting of preoperative therapy if one believes that less viable tumor tissue may be present around the vessels after chemoradiation.

Observations from these early radiology studies and fine tuning of BRPC criteria have led experts to objectively define tumors with $\leq 180^{\circ}$ of arterial abutment

(≤50% circumferential involvement) as BRPC. As discussed below, these tumors with arterial involvement require a multimodality approach to their disease to help achieve an R0 margin resection.

Short-Segment Interface of Any Degree Between Tumor and HA

Limited or "short-segment" encasement of the common hepatic artery or the proper hepatic artery, typically at the gastroduodenal artery origin, is also included in the definition of BRPC. These patients are often candidates for vascular reconstruction with grafting. In selected patients, segmental resection with primary end-to-end anastomosis can be performed to achieve an R0 resection.

An Interface Between the Tumor and SMV–PV ≥180° of the Vein Circumference OR a Short-Segment Occlusion of the SMV–PV with Option for Reconstruction

In most patients, occlusion of the SMV or SMPV confluence by tumor suggests SMA or celiac axis involvement as well, given the proximity of the SMV to the SMA. Unfortunately for most patients, occlusion of the SMV precludes surgery since there is no patent vessel above and below the occlusion to allow interposition grafting. In a small select group of patients, short segment occlusion of the SMV with sufficient venous flow above and below the occlusion may allow them to be categorized as having BRPC. In a retrospective study recently published by Tseng and colleagues from MDACC, 141 patients who underwent vascular resection (VR) (these were not all segmental occlusions) at the time of PD were compared with patients who underwent standard PD without vascular resection [26]. Median survival was similar in both groups (23.4 months in the group that required VR and 26.5 months in the group that underwent standard surgery; $P = 0.177$). Seventy percent of patients in both groups received preoperative chemoradiation. Patients with R0 versus R1 margin had similar survival, and the authors believe this was due to the use of neoadjuvant therapy as well as meticulous margin analysis (i.e., all the R1 margin resections were truly R1 and not R2 resections). Considering the results of this retrospective study, these patients also benefit from preoperative therapy and the role of radiation is less clear.

Expanded Criteria for Borderline Resectable Pancreatic Cancer: MDACC Types B and C

Katz and colleagues have described two additional subsets, types B and C, which define borderline resectable cancer beyond the tumor-vessel orientation and anatomic criteria [27]. Most physicians have come across patients with localized pancreatic cancer who are not ready for immediate surgery. Some of these patients have subtle indeterminate subcentimeter liver or omental lesions that are suspicious for metastatic disease but the lesions are too small for FNA- biopsy or additional imaging tests (PET-CT or MRI). These patients fit the MDACC type B definition of BRPC. Type B patients may have a technically resectable or a borderline resectable

primary tumor as defined on CT images. Another subset of patients are those who have associated medical comorbidities that need further time consuming evaluation or they have a poor performance status (typically ECOG 3), albeit reversible but still risky to proceed with up-front surgery. A good example of this presentation is a patient who has had a significant decline in nutrition and performance status in the presence of obstructive jaundice and cholangitis and a steady improvement is expected after biliary decompression, better nutritional supplementation, and supportive care. This constitutes MDACC Type C subset, and patients in this category may also have had a radiographic potentially resectable or a borderline resectable primary tumor.

Role of Preoperative Therapy in Patients with Borderline Resectable Pancreatic Cancer (Types A, B, C)

Adjuvant and metastatic trials inform preoperative trials: Current trials of adjuvant therapy have clearly demonstrated a small but absolute benefit of systemic therapy for the prevention of disease recurrence. The assumption is that this benefit derives from treatment of microscopic disease that is neither clinically or radiographically apparent. The ESPAC and CONKO results have established fluoropyrimidine and gemcitabine-based chemotherapy regimens as effective in the adjuvant setting [2, 27–30]. Additionally, early preoperative trials in resectable pancreatic cancer were the building blocks to augment the rationale for neoadjuvant therapy in pancreatic cancer and for the management of BRPC [31–34]. Finally, prospective data from metastatic disease is extrapolated into locally advanced and from there, BRPC neoadjuvant setting.

***Rationale in BRPC*:** The rationale for pursuing preoperative treatment for a patient with BRPC is similar to patients with potentially resectable pancreatic cancer although with a greater emphasizes on maximizing R0 resection. Additional justification for preoperative therapy includes treating micro metastatic disease early, giving majority of the "adjuvant" therapy in a "neoadjuvant" setting when it is better tolerated. Using this approach to gauge the aggressiveness of the cancer selects patients for surgery who have the greatest likelihood of a favorable postoperative outcome especially given the morbid nature of the surgery. Data also suggests that preoperative chemoradiation may decrease the incidence of pancreaticojejunal anastomotic fistula, a common complication following PD or distal pancreatectomy. Therefore, although the sequencing and duration of preoperative treatment modalities remain elusive, most agree that a treatment schema that incorporates systemic chemotherapy with/without chemoradiation is the optimal strategy for BRPC, and this notion has been embraced by several institutions and high volume pancreatic cancer centers. At MDACC, for nonprotocol patients, patients with BRPC are presented in the multidisciplinary conference with radiology review of the pancreas protocol optimized CT; patients are categorized as borderline resectable types A, B, C, or a combination of these. A restaging CT scan is reviewed after approximately 8–10 weeks of systemic therapy. Patients with radiographic response or a

biochemical response in the presence of stable disease are candidates for more systemic therapy followed by chemoradiation or may proceed to chemoradiation or surgery [35]. If radiated, after a break of 4–6 weeks from their radiation therapy, patients who continue to show disease stability or response are candidates for surgery. The duration of systemic therapy and role of chemoradiation depends on the concern for micrometastatic disease (CA19-9, indeterminate extrapancreatic lesions) and confidence to proceed with margin negative resection (RT in select patients, if concern for R1 margin).

Retrospective Preoperative Data for Management of BRPC (Type A)

Katz et al. published the first large retrospective report of BRPC; 160 patients were identified as having BRPC and of these, 125 (78%) received preoperative therapy with mostly chemotherapy followed by chemoradiation and 66 (41%) underwent PD. [7] Twenty-seven percent (18 of 66) required vascular resections and in 94% of the patients this was an R0 resection. The median survival was 40 months for patients who underwent preoperative therapy followed by surgery and 13 months for patients who did not undergo PD ($p < 0.001$). When compared to patients who had an increase in their serum CA19-9 level over the course of induction therapy, patients whose serum CA19-9 fell were more likely to undergo pancreatectomy. The percent change in CA19-9 over the course of neoadjuvant treatment was associated with overall survival. When compared to patients who had a $> 50\%$ decrease in serum CA19-9, patients with an increase in serum CA19-9 had a greater than twofold risk of death (HR $= 2.4$, $p = 0.020$). These numbers are small though suggest that CA19-9 along with the imaging studies and host factors play a role in deciding resectability.

Chun et al. [36] reported the impact of neoadjuvant chemoradiation on margin negative resection in borderline resectable cases involving the portal or superior mesenteric vein (PV-SMV). They compared 74 preoperatively treated patients to 35 that received upfront surgery. Of those treated, 78% received gemcitabine-based chemoradiation while 22% received 5-FU based chemoradiation. They found improved survival with chemoradiation in patients with unilateral involvement of the PV-SMV (Ishikawa type II and III); however, there was not a significant survival benefit with bilateral involvement (Ishikawa type IV and V). Overall, preoperative therapy and margin negative resection status both were associated with improved survival in these cases involving the PV-SMV.

Stokes and colleagues [37] evaluated patients with borderline resectable disease by the MDACC classification who were treated with preoperative capecitabine with radiation. Among the 40 B.P. patients, 85% completed therapy and 16 underwent resection. R0 resection was achieved in 75% of surgical cases. The authors conclude that capecitabine-based chemoradiation is well tolerated and effective in selecting patients most likely to benefit from surgery.

Chuong and colleagues [38] reported sequential induction with 3 cycles of chemotherapy followed by SBRT in a cohort of BRPC patients. About 66% of patients received a combination of gemcitabine, docetaxel, and capecitabine (GTX), and the majority received gemcitabine-based therapy. Of those treated, 56% went to

surgery and 97% of those achieved an R0 resection. Among these, three patients had a pathologic complete response (pCR) and one had a near pCR.

Several small trials have reported positive outcomes with FOLFIRINOX in the preoperative setting [39–40]. Paniccia reported on a small retrospective cohort of patients who received FOLFIRINOX. Approximately half received only chemotherapy while the rest received chemotherapy followed by chemoradiation. Approximately, 90% of patients completed chemotherapy. About 85% underwent resection and all those patients achieved R0 resection. A systematic review of FOLFIRINOX in BRPC and locally advanced pancreatic cancer suggests a response rate of ~25–30% in the primary tumor although this is mostly investigator reported data [41].

These retrospective single institution studies yield valuable information, although have several limitations including unclear BRPC criteria and multiple neoadjuvant approaches. As such, it is difficult to determine what components of chemotherapy or chemoradiation are providing the most benefit. Fortunately, results from small prospective trials are emerging and several ongoing larger randomized controlled trials will further help evaluate the best sequence and duration of preoperative regimens in a background of homogenous BRPC population.

Prospective Trials in BRPC and Current Ongoing Studies

Select gemcitabine-based prospective trials: Sahora et al. published the results of two separate phase-II studies with neoadjuvant gemcitabine plus either oxaliplatin or docetaxel. In the gemcitabine and oxaliplatin (GemOx) study [42], patients received 6–9 weekly doses of GemOx with restaging and surgical exploration if evidence of response on imaging or clinically. Of the 15 patients who were classified as borderline resectable at enrollment, 47% underwent surgical exploration. R0 resection rate was 69%, and median survival was 22 months for resected versus 12 months for unresected patients. The gemcitabine and docetaxel (GemTax) trial [43] treated patients with 8 weeks (2 cycles) of GemTax prior to restaging. Patients with partial response or stable disease with improved clinical condition were taken for surgical exploration. Of the 12 patients with BRPC at study entry, 7 (58%) underwent surgical exploration and ultimately 4 (33%) were resected with curative intent. The overall R0 resection rate was 87%. Median survival among resected versus unresected patients was 16.3 months versus 12.2 months, respectively.

A separate phase II study examined the role of neoadjuvant dose-dense gemcitabine and capecitabine (GX) in locally advanced pancreatic cancer [44]. Treatment typically consisted of 2 weeks of weekly gemcitabine and daily capecitabine on a 3 week cycle (average number of treatment cycles was three). Per protocol, patients were classified as BRPC based on NCCN criteria, and 18 B.P. patients were enrolled along with 23 LAPC patients. A total of 11 (61%) underwent surgical resection and 9 of 11 (82%) were R0 resections. Interestingly, the authors also analyzed patients based on Asian Pancreatobiliary Cancer Center (APBCC) criteria, which results in 33 out of 43 patients being classified as borderline resectable. With broader inclusion criteria, a smaller proportion of patients (46%) underwent resection, yet a greater number, 13 of 15 (87%), were R0 resections. The median survival of resected

patients was 23.1 months compared with 13.4 months in unresected patients. This trial also demonstrates the importance of standardization of BRPC criteria.

In another study, 35 B.P. patients were treated with combination S-1 with gemcitabine. Twenty seven patients had no evidence of distance metastatic disease at time of resection and had a median survival of 35 months compared with 10 months for those with unresectable or metastatic disease [45]. The internal variability of results based on the borderline resectable classification system demonstrates the challenge of comparing results between trials.

Predominant chemoradiation trials: Mehta and colleagues conducted the earliest prospective trials of preoperative chemoradiation in patients with borderline resectable characteristics. Specifically, they enrolled patients with pancreatic adenocarcinoma who had >1 cm of tumor abutment, but <180° involvement of the PV, SMV, or SMA [46]. Patients received protracted 5-FU infusion with concurrent radiation totaling between 50.4 and 56 Gy. Of those treated, 60% underwent surgery, all with R0 resection and had a median survival of 30 months compared with 8 months for the remaining unresected patients.

Landry et al. reported on a randomized phase II trial comparing neoadjuvant regimens although trial was closed for poor accrual (total 21 patients) [47]. In Arm A, 10 patients received gemcitabine-based chemoradiation, and in Arm B, 11 patients received induction chemotherapy using gemcitabine + cisplatin +5-FU followed by chemoradiation with 5-FU. Three patients in Arm A and two patients in Arm B were resected. The median survival of resected patients was 26.3 months. All patients received adjuvant gemcitabine for five cycles.

A study by Takahashi et al. investigated a regimen of gemcitabine-based chemoradiation followed by gemcitabine in resectable and borderline resectable patients [48]. Of 80 B.P. patients, resection rate was 54%, and among those resected 34% were alive at 5 years. Notably, distant and peritoneal recurrence was significantly higher in the BRPC group than the baseline resectable cohort. Given higher rates of recurrence, borderline resectable patients may benefit from higher intensity chemotherapy regimens in the neoadjuvant setting.

Another trial of chemoradiation therapy with S-1 enrolled 28 patients, 25 of whom completed treatment. About 24 (85.7%) underwent surgical resection and all achieved R0 resection [49]. The large phase III trials of S-1 have taken place in Japan and there are concerns about how the toxicity profile, particularly in Western populations, may limit utilization of this drug in the Western continents. These results are encouraging, and trials of S-1 compared with the more aggressive and established neoadjuvant regimens are warranted.

FOLFIRINOX-based preoperative trials: FOLFIRINOX is commonly used in locally advanced pancreas cancer and given that BRPC bridges the continuum of resectable and LAPC, even without prospective data, the regimen is frequently used in patients with good performance status without biliary complications. In a systematic review of FOLFIRINOX trials, there were patients with BRPC and LAPC treated with this regimen for advanced localized PDAC [50]. Thirteen studies comprising 689 patients, of whom 355 (52%) patients had LAPC. Eleven studies, comprising 315 patients with LAPC, reported survival outcomes and were eligible

for patient-level meta-analysis. Median overall survival from the start of FOLFIRINOX ranged from 10.0 months (95% CI 4.0–16.0) to 32.7 months (23.1–42.3) across studies with a pooled patient-level median overall survival of 24.2 months (95% CI 21.7–26.8). In eight studies, 154 (57%) of 271 patients received radiotherapy or chemoradiotherapy after FOLFIRINOX. The pooled proportion of patients who received any radiotherapy treatment was 63.5% (95% CI 43.3–81.6, I(2) 90%). The proportion of patients who underwent surgical resection for LAPC (likely including all with BRPC criteria) ranged from 0% to 43%. In 12 studies, 91 (28%) of 325 patients underwent resection after FOLFIRINOX. R0 resection was reported in 60 (74%) of 81 patients. Given the heterogeneity, selection bias, improvements in perioperative care, and surgical skills, the added benefit of any specific modern regimen over another is difficult to interpret although all would agree that multicytotoxic therapy has likely moved the therapeutic needle compared to gemcitabine alone in the preoperative setting [51].

The only published prospective data using strict criteria is from Alliance A021101 intergroup trial for BRPC with preoperative FOLFIRINOX followed by capecitabine-based chemoradiation [52]. Twenty-nine patients were registered and 22 initiated therapy. Although 14 of the 22 patients (64%) had grade 3 or higher adverse events, 15 of the 22 patients underwent pancreatectomy. About 80% required vascular resection, 14 (93%) had microscopically negative margins, 5 (33%) had specimens that had <5% residual cancer cells, and 2 (13%) had specimens that had pathologic complete responses. The median overall survival of all patients was 21.7 months from registration.

Novel prospective trials: BRPC is an attractive platform for novel agents added to cytotoxic therapy since the resected tissue allows for extensive pharmacodynamics and pharmacogenomics and whole tumor mapping studies not feasible with core biopsies. An example is PF-04136309, a chemokine receptor type 2 (CCR2) antagonist which was studied in BRPC [53]. Activation of CCR2 mobilizes monocytes and macrophages from the bone marrow to infiltrate malignant tumors. These inflammatory monocytes appear to have tumor-promoting immunosuppressive properties. Inhibition of CCR2 with PF-04136309 resulted in enhanced antitumor immunity, decreased tumor growth, and reduced metastasis in preclinical models. A recent phase 1B trial with FOLFIRINOX and CCR2 antagonist in BRPC patients showed encouraging results with PF-04136309.

Considerations for Preoperative Therapy for BRPC

Biopsy and stent evaluation: Patients with BRPC need a cytologic diagnosis of cancer via EUS-guided FNA biopsy prior to initiating therapy. The risk of occlusion of plastic stents increases with a longer period of preoperative therapy, and biliary stent related concerns need vigilant care [54–55]. In a clinical trial of 79 patients undergoing chemotherapy with gemcitabine in combination with cisplatin followed by gemcitabine-based chemoradiation, at least one stent exchange was necessary in 46 (75%) of the 61 patients who entered the protocol with a plastic biliary stent and

self-expandable metal stents which ultimately were placed in 36 (46%) of 79 patients [33]. In the study by Katz evaluating the borderline resectable cancers, of the 125 patients who underwent a complete restaging evaluation, endobiliary stent exchange was necessary in 19 (15%) due to stent occlusion or cholangitis and most patients had a metal stent placed at the initiation of therapy [7]. Additionally, covered stents are associated with decreased tumor ingrowth and improved patency and are therefore preferred to uncovered stents [56–57].

Prehabilitation: Prehabilitation is gaining significant interest in health care and refers to enhancing a patient's functional capacity prior to medical or surgical intervention [58]. While the term originally applied to improving physical capacity, most prehabilitation programs are multidimensional and address debilitation, improving nutrition, and optimizing comorbid and psychosocial conditions. Prehabiliation therapy has shown substantive improvements in rates of postoperative recovery in colorectal cancer patients [59–60] and chemotherapy tolerance in breast cancer patients [61]. It is increasing being recognized that patients with BRPC and marginal performance status and/or reversible comorbidities are at higher risk of poor outcomes [62]. This is especially important when months of preoperative therapy is implemented with the eventual goal being to proceed with a large abdominal surgery and likely, additional systemic therapy on recovery. Currently, prospective trials are studying the role of individualized exercise programs, personal nutrition plans made by registered dieticians, medical or geriatric consultation/optimization, and psychosocial evaluations for PDAC patients with an emphasis on elderly patients [63–65]. The outcome measures include following postoperative surgical complications, length of hospitalization, quality of life, and other important results, including postoperative pain level and returning to work and leisure activities. Through creative prospective randomized trials, we can learn the impact of these measures and gauge its effect on the immune milieu in blood and tumor tissues.

Setting expectations: Forty to 60% of all patients who start preoperative therapy for BRPC eventually proceed to pancreatectomy [66]. Many relapse within 3 years of surgery and cure rate remains low. It is important to address patient expectations early and revisit goals at presentation and each restaging to minimize disappointment and caregiver stress and burnout. Most neoadjuvant programs are aggressive and prolonged (several months), and complications, particularly in high-risk patients, may interrupt or necessitate a change in plans [64].

Biomarkers and BRPC

With the current available therapies, a minority of patients achieve an excellent response to neoadjuvant therapy (<10% viable tumor cells) and eventual cure. It is difficult to identify these patients a priori, and the radiographic responses and CA19-9 serve as poor surrogates with their limitations, especially in a disease that at presentation is systemic in most patients. BRPC remains a heterogeneous entity, and prognostic or predictive biomarkers are urgently warranted.

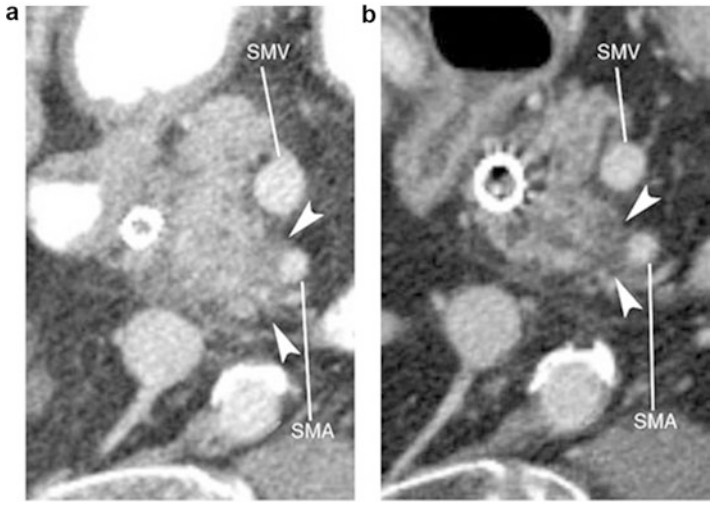

Fig. 1 Pre- and post-treatment CT scans of a 78-year-old patient presenting with tumor abutting the SMA. (**a**) Pretreatment scan shows tumor (*white arrowhead*) involving the SMA for approximately 180°. Patient's preoperative CA19-9 was 359 U/ml. Patient was treated with gemcitabine and nab-paclitaxel for two cycles and followed by gemcitabine-based chemoradiation (50.4 Gy); (**b**) after chemotherapy and chemoradiation, despite reduction in the size of the hypodense tumor, arterial abutment was still present though there was a decline in the CA19-9 to 40 U/ml. Approximately 5 months after her initial visit, patient underwent pancreaticoduodenectomy. Pathology revealed a residual infiltrating moderately differentiated adenocarcinoma of the head of the pancreas (2.0 cm), a treatment effect was seen, with 20–30% viable tumor. Proximal gastric, distal duodenal, and retroperitoneal resection margins were negative for tumor (R0 resection), and the tumor was 1.0 cm from the retroperitoneal margin. Seventeen regional lymph nodes were removed and all were negative for micrometastatic disease. Patient received adjuvant chemotherapy with single agent gemcitabine. The patient is 40 months out from completion of her treatment with no radiographic evidence of local recurrence or metastases

The role of SMAD-4 as a biomarker of disease progression and metastases needs to be studied in a prospective setting and hence, guide if it facilitates the discussion surrounding the role preoperative radiation therapy in select patients [67]. Iacobuzio-Donahue et al. performed rapid autopsies on 76 PDAC patients and at autopsy, 30% of patients died with locally destructive disease, and 70% died with widespread metastatic disease [68]. Tumor SMAD4 immunolabeling status harvested at autopsy correlated with the presence of widespread metastasis but not with locally destructive tumors ($P = 0.007$). The authors concluded that SMAD4 intact cancers may be more locally destructive and hence these patients benefit from loco-regional therapies, whereas SMAD4 deleted cancer represents an aggressive metastatic biology. Boone et al. studied 117 patients who underwent pancreaticoduodenectomy with venous resection [69]. Sixty had sufficient specimens available for SMAD4 staining. SMAD4 loss was observed in 70% of resections and was associated with earlier time to metastatic disease. Preoperative SMAD4 loss correlated well with postoperative

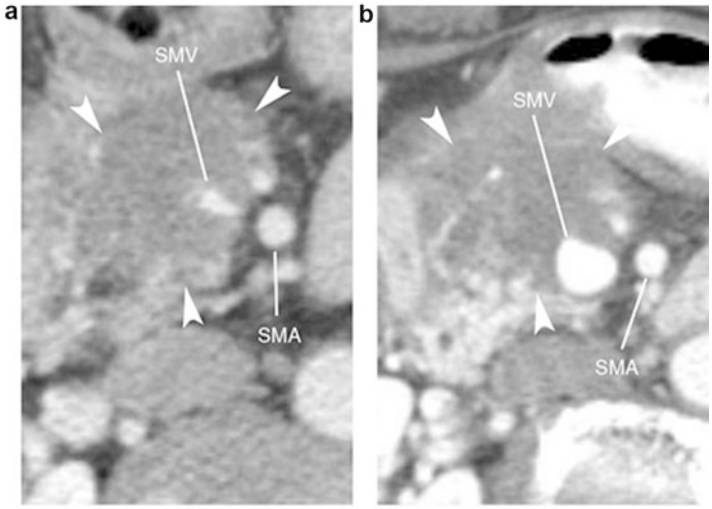

Fig. 2 Pre- and posttreatment CT scans of a 70-year-old patient presenting with segmental venous near occlusion. (**a**) Pretreatment scan shows a hypodense mass (*white arrowheads*) involving the head of the pancreas with marked narrowing of the SMV. Patient's baseline CA19-9 was 71 U/ml. Patient was treated with gemcitabine and cisplatin every 2 weeks for 4 infusions followed by capecitabine-based chemoradiation (50.4 Gy); (**b**) after chemotherapy and chemoradiation, there was significant improvement in the caliber of the SMV without much change in the tumor size and the CA19-9 was 27 U/ml. At surgery, tumor was found extending substantially to the left of the SMA for a distance of more than a centimeter. This process extended to the right to entirely encase the tributaries to the SMV such that there would be no access to a reasonable trunk of the SMV to enable venous resection. Patient is currently 24 months out from her attempted surgery and undergoing chemotherapy for progressive disease manifested by carcinomatosis

staining and was associated with 6 times higher likelihood of developing metastases. The authors concluded that preoperative SMAD4 status may be considered as one of several factors when selecting patients most likely to benefit from aggressive surgery. A recent prospective trial, RTOG 4201, in patients with locally advanced PDAC, using SMAD4 as a stratification factor, randomized patients to systemic therapy followed by RT (50.4 vs. 60 Gy) vs. systemic therapy alone – unfortunately, it was closed early due to poor accrual. Alliance 021501 is a randomized phase II trial of combination chemotherapy (mFOLFIRINOX) with or without hypofractionated radiation therapy before surgery and although the sample size is small, SMAD4 biomarker data from this study may further help define the role of SMAD4 and RT in BRPC.

Koay and colleagues have demonstrated the interpatient variability in the delivery of gemcitabine as well as in the mass transport properties of tumors as measured by computed tomography (CT) scans [70–71]. They developed a volumetric segmentation approach to measure mass transport properties from the CT scans of PDAC patients and tested interobserver agreement with this new methodology. The

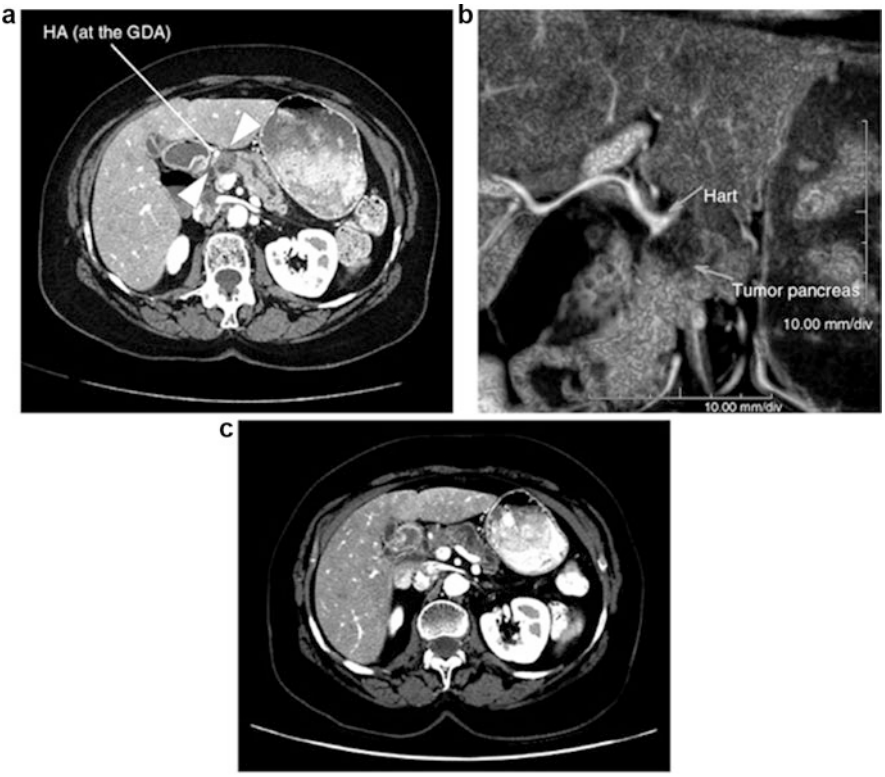

Fig. 3 Pre and posttreatment CT scans of a 74-year-old patient presenting with short-segment involvement of the hepatic artery (*HA*) at the level of the gastroduodenal artery (*GDA*). (**a**) Pretreatment scan shows tumor (*white arrowhead*) involving the HA; (**b**) the scans showed a 1.6 cm hypodense mass that was inseparable from the RHA and proximal GDA at the bifurcation as seen in the coronal view. The left HA was noted to arise from the left gastric artery. Patient was treated with capecitabine-based chemoradiation for a total dose of 50.4 Gy in 28 fractions; (**c**) postchemoradiation scan shows persistent hepatic arterial involvement. Patient underwent an R0 pancreaticoduodenectomy with resection of the hepatic artery with primary repair. Pathology showed a residual moderately differentiated ductal adenocarcinoma (1.7 cm) with perineural and lymphovascular invasion. Tumor was 1.1 cm from the retroperitoneal margin. Eighteen regional lymph nodes were removed and all were negative for micrometastatic disease. Patient completed adjuvant single agent gemcitabine chemotherapy, and interim CT scan showed no evidence of disease 1 year following pancreaticoduodenectomy

quantitative method to derive transport properties from CT scans demonstrated $<5\%$ difference in gemcitabine prediction at the average CT-derived transport value across observers. The authors concluded that with further validation as a biophysical imaging marker, transport properties of tumors (derived from standard of care CT images) may be useful in patient selection for therapy and prediction of therapeutic outcome (Figs. 1, 2, and 3).

Conclusion

Determining resectability of the primary pancreas tumor is essential to the initial staging evaluation. There is significant progress made in defining BRPC and planning early small prospective trials. The heterogeneity presents a challenge and all BRPC is not created equal and may not benefit from an identical therapy sequence. As preoperative systemic and loco-regional therapies improve, the need to use prognostic and predictive markers in BRPC will be vital to decision-making. It is imperative to plan innovative trials and to evaluate the role of liquid biopsies (exoDNA, ctDNA) and radiogenomics in this setting.

Cross-References

- ▶ Clinical Decision-Making in Pancreatic Cancer
- ▶ Development of Novel Therapeutic Response Biomarkers
- ▶ Neoadjuvant Chemotherapy in Pancreatic Cancer
- ▶ Pancreatic Adenocarcinoma: CT and PET/CT
- ▶ Therapeutic Endoscopy in the Management of Pancreatic Cancer

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New Japanese Classification of Pancreatic Cancer

Shuji Isaji, Yasuhiro Murata, and Masashi Kishiwada

Contents

Abstract

Background: The Japanese classification of pancreatic cancer, seventh edition, has been released by Japan Pancreas Society (JPS) in July 2016.

Methods: Revision concepts and major revision points of the seventh edition of Japanese classification of the pancreatic cancers were reviewed.

Results: The principal points of revision are as follows:

Conflicts of Interest: Shuji Isaji, Yasuhiro Murata, and Masashi Kishiwada declare that they have no competing interests.

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1. Definition of the parts of the pancreas.
2. T category and stage grouping: consistency with those of the UICC seventh edition was obtained.
3. Reappraisal of anatomy of extrapancreatic nerve plexuses.
4. N category: classification based on numbers of lymph nodal metastasis among the regional lymph nodes; N1a, metastasis in one to three regional lymph nodes; and N1b, metastasis in four or more regional lymph nodes.
5. Histopathological classification which is consistent with the WHO classification.

The following new items have been added: (1) diagnostic guideline of tumor extension and lymph node metastasis based on multidetector CT (MD-CT), (2) objective criteria defining resectability status only based on the findings of MD-CT, (3) cytopathology guideline, and (4) criteria of histological response to drug therapy and/or radiotherapy.

Conclusion: The revised seventh edition of JPS pancreatic cancer classification focuses on establishing consistency to UICC seventh edition, while originality of JPS classification is maintained.

Keywords
Pancreatic cancer · Japanese classification · Staging system · UICC/AJCC staging system

Introduction

The purpose for establishment of pancreatic cancer classification is to make rules and guidelines so that clinicians and pathologists can compare and discuss collected cancer status and clinical outcomes based on a common criteria. As for the classification of pancreatic cancer, the first edition of the Japanese edition of the General Rules for the Study of Pancreatic Cancer was released in 1980 by the Japan Pancreas Society (JPS). The sixth edition by JPS was published in 2009 [1], and at the same year, the Union Internationale Contre le Cancer (UICC) [2] published its seventh edition. Two classifications adopted TNM classification, but they had been quite different in T category, N category, and Staging system, constituting obstacles to compare status and clinical outcomes of pancreatic cancer patients between Japan and western countries. Therefore, the revision committee of JPS (Isaji S. is the chairperson), which consists of 12 pancreatic surgeons, 4 gastroenterologists, 7 pathologists, 1 radiologist, 1 anatomist, and 1 doctor from Pancreatic Cancer Registry Committee in JPS, started its work in April 2013, and the seventh edition has been published in July 2016. The current revision by JPS focuses on establishing consistency between the Japanese and UICC classifications; however, originality of JPS classification, which is more precise and contains more information, is maintained.

In the seventh edition, major revisions have been carried out by comparing the sixth edition in the following points:

1. Definition of the portions of the pancreas: the border between the pancreatic body and tail is defined as the left side line of the abdominal aorta.
2. T category: consistency with that of the UICC seventh edition.
3. Reappraisal of anatomy of extrapancreatic nerve plexuses.
4. N category: new classification based on numbers of lymph nodal metastasis among the regional lymph nodes.
5. (5)Stage grouping: consistency with UICC staging system.
6. Histopathological classification: consistency with the WHO classification.

In the current revision, the following new items have been added: (1) criteria of diagnosis for T category based on MD-CT, (2) criteria of diagnosis for lymph nodal metastasis based on MD-CT, (3) criteria defining resectability, (4) cytopathology guideline, and (5) criteria of histological response to drug therapy and/or radiotherapy.

Definition of Parts of the Pancreas

The definition of portion of the pancreas is shown in Fig. 1. The border between pancreatic head and body is defined as the left side of the superior mesenteric vein (SMV) and portal vein (PV). The neck of the pancreas (a part anterior to the SMV and PV) and uncinate process are included in the pancreatic head. In the sixth edition by JPS, the boundary between the body and tail of the pancreas was the line dividing the distal pancreas into two equal halves. In the seventh edition, its boundary is revised as the left border of the aorta, which is the same as UICC classification. This is attributed to the fact that pancreatic cancer arising from the site between the left side of SMV/PV and left border of the aorta tends to be frequently unresectable due to the involvement of celiac axis (CA) and/or superior mesenteric artery (SMA).

Category of Tumor Extension (T)

Comparison of T categories between JPS sixth and seventh, UICC seventh edition, and the American Joint Committee on Cancer (AJCC) eighth edition is summarized in Table 1. T1 and T2 are almost the same in the three (JPS sixth and seventh and UICC seventh) classifications. T3 and T4 are quite different between JPS sixth edition and UICC seventh edition. In JPS seventh edition, however, T3 and T4 are the same as those of UICC seventh edition. Clinically, the involvement of CA and SMA is clearly defined as tumor with contact or invasion of arterial wall based on dynamic CT findings. Among T1, T1 is divided into subclassifications: T1a, 5 mm or less; T1b, more than 5 mm but 10 mm or less; and T1c, more than 10 mm but 20 mm or less.

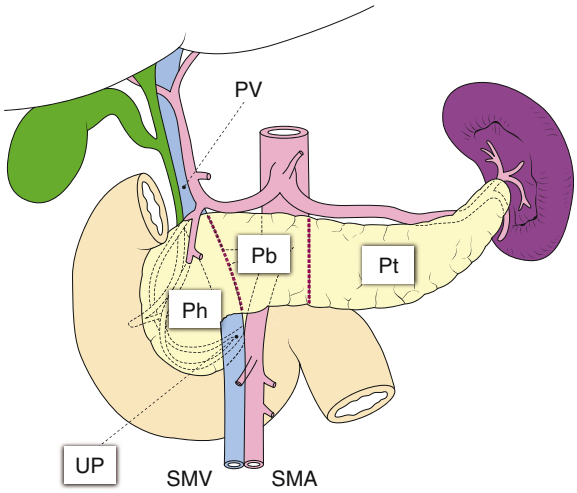


Fig. 1 Portion of pancreas in Japanese classification of pancreatic cancer, seventh edition (Kanehara & Co., Ltd., Tokyo, Japan). The border between pancreatic head and body is defined as the *left side* of SMV and PV. The neck of the pancreas (a part above SMV and PV) and the uncinate process are included in the pancreatic head. The border between pancreatic body and tail was defined as *left side line* of abdominal aorta. *Ph* pancreatic head, *Pb* pancreatic body, *Pt* pancreatic tail, *PV* portal vein, *SMA* superior mesenteric artery, *SMV* superior mesenteric vein, *UP* uncinate process

The AJCC proposed changes for T staging in its eighth edition [3]. These changes have focused on improving the reproducibility of T stage, decreasing the percentage of tumors designated as T3, because the term "extension beyond the pancreas" for description of T3 in AJCC seventh edition has been thought to be potentially inconsistent between pathologists and the T stage defined as such was not found to have any correlations with survival [4]. A revised T stage protocol was devised that defined pT1 as 2 cm or smaller, pT2 as >2–4 cm, and pT3 as larger than 4 cm. The multi-institutional comparative study proved that the proposed cutoff points for T stage were statically valid, and its utilization was more reproducible between institutions and pathologists [5].

Reappraisal of Anatomy of Extrapancreatic Nerve Plexus

From the third to sixth edition by JPS, the extrapancreatic nerve plexuses, which were originally defined in Japan according to the literature reported by Yoshioka et al. [6], were divided into seven parts of the plexus: PLphI, pancreatic head plexus I; PLphII, pancreatic head plexus II; PLsma, SMA plexus; PLcha, CHA plexus; PLhdl, plexus within the hepatoduodenal ligament; PLspa, SPA plexus; and PLce, celiac plexus. Several problems were pointed out for the scheme which had been used until the sixth edition (Fig. 2). First, PLphI and PLphII were drawn on the same

Table 1 Comparison of T categories between JPS sixth and seventh, UICC seventh edition, and AJCC eighth

	JPS sixth edition (2009)	JPS seventh edition (2016)	UICC seventh edition (2009)	AJCC eighth edition (2016)
T1	Tumor limited to pancreas, 2 cm or less in greatest dimension	Tumor limited to pancreas, 20 mm or less in greatest dimension	Tumor limited to pancreas, 2 cm or less in greatest dimension	Maximum tumor diameter <=2 cm
		T1a: 5 mm or less		
		T1b: more than 5 mm but 10 mm or less		
		T1c: more than 10 mm but 20 mm or less		
T2	Tumor limited to pancreas, more than 2 cm in greatest dimension	Tumor limited to pancreas, more than 20 mm in greatest dimension	Tumor limited to pancreas, more than 2 cm in greatest dimension	Maximum tumor diameter >2 cm < =4 cm
T3	Tumor that has extended into any of the following: bile duct (CH), duodenum (DU), peripancreatic tissue (S, RP)	Tumor extends beyond pancreas, but without involvement of celiac axis or superior mesenteric artery	Tumor extends beyond pancreas, but without involvement of celiac axis or superior mesenteric artery	Maximum tumor diameter > 4 cm
T4	Tumor that has extended into any of the following: adjacent large vessels (PV, A), extrapancreatic nerve plexus (PL), other organs (OO)	Tumor involves celiac axis or superior mesenteric artery (Tumor contant or involvement on the dynamic CT findings)	Tumor involves celiac axis or superior mesenteric artery	Tumor involves the celiac axis or the superior mesenteric artery (unresectable primary tumor)

CH distal bile duct invasion, *DU* duodenal invasion, *S* serosal invasion, *RP* retropancreatic tissue invasion, *PV* portal venous system invasion, *A* arterial system invasion, *PL* extrapancreatic nerve plexus invasion, *OO* invasion of other organs

cross section as shown in Fig. 2a. However, it is not correct to draw these plexuses on the same cross section, because PLphI is located at the cranial side and PLphII is located at the caudal side. Second, PLphI and PLph2 were drawn just like thick nerve bundles as shown in the yellow colored site in Fig. 2b. Third, the third and fourth portion of the duodenum is located at the right side of the SMA, which is not correct anatomy (Fig. 2b).

In JPS seventh edition, anatomy of the extrapancreatic nerve plexuses had been reappraised based on cadaveric anatomical findings reported by Yi et al. [7] and several discussions between anatomists and surgeons, and finally the revision committee of JPS decided to make a new scheme as shown in Fig. 3. The current

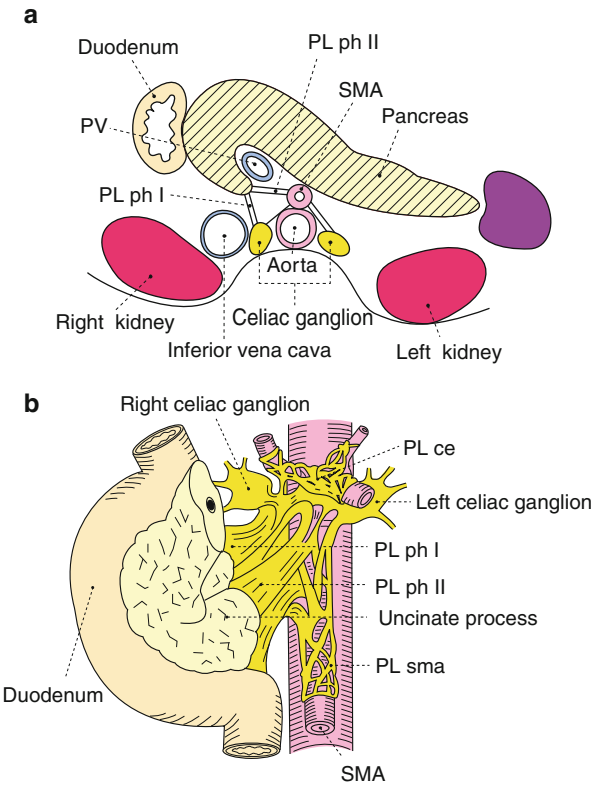


Fig. 2 Anatomical scheme of the extrapancreatic nerve plexuses including pancreatic head nerve plexuses (PLph) in Japanese classification of pancreatic cancer, sixth edition (Kanehara & Co., Ltd., Tokyo, Japan). (**a**) Pancreatic nerve plexuses (cross-sectional diagram). (**b**) Extrapancreatic nerve plexuses. *PV* portal vein, *SMA* superior mesenteric artery, *PLphI* pancreatic head plexus I, *PLphII* pancreatic head plexus II, *PLCe* celiac plexus, *Plsma* superior mesenteric arterial plexus

reappraisal of anatomy of extrapancreatic nerve plexuses has clarified that nerves within PLphI and PLphII are much less and thinner than those previously considered. Although they can be actually defined as nerve plexus from the perspective that sympathetic nerve and parasympathetic nerve cross and make a network of nerve, the membranous structures which are drawn as PLphI and PLphII have been proven to include not only nerve tissue but also fibrous tissue, capillaries, and fat tissue. Taken together with operative finding and cadaveric anatomical finding, PLphI is a region which mainly includes nerve tissue distributed to the dorsal surface of pancreatic head from the celiac plexus, while PLphII includes nerve tissue distributed to the uncinate process from the SMA plexus. Because PLphI and PLphII are frequently involved by pancreatic head carcinoma and invasion of these areas is the main cause of incomplete resection [8–10], it is very important to understand the anatomy of extrapancreatic nerve plexus for making proper diagnosis of plexus nerve invasion based on MD-CT and for determining the level of plexus nerve dissection during pancreatectomy.

In the seventh edition, the term “mesopancreas” is not adopted, because its concept and anatomical definition remain uncertain. Gockel et al. [11] has defined a membranous structure between SMA and pancreatic head as mesopancreas, which contains nerve tissue, capillaries, fibrous tissue, and fat tissue.

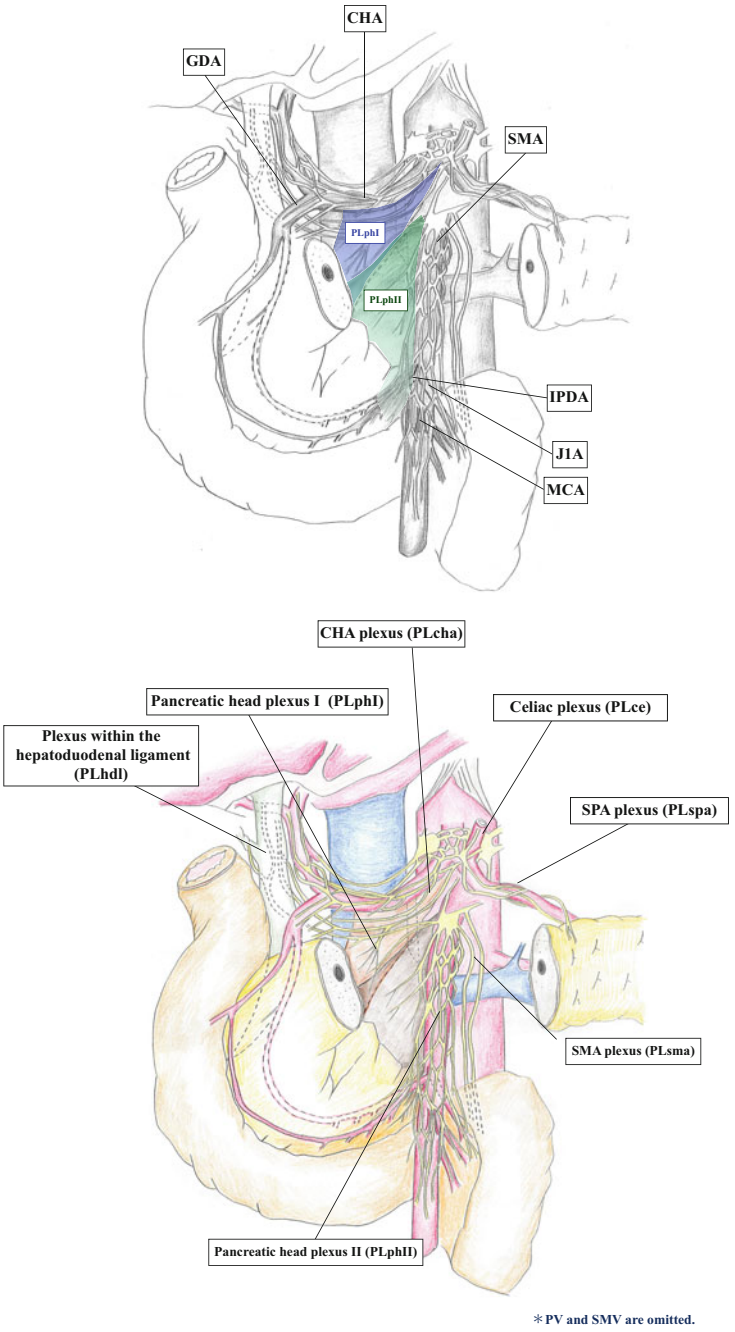


Fig. 3 Anatomical scheme of the extrapancreatic nerve plexuses including pancreatic head nerve plexuses (PLph) in Japanese classification of pancreatic cancer, seventh edition (Kanehara & Co., Ltd., Tokyo, Japan)

Mesopancreas seems to be consistent with PLphII, but they did not mention PLPhI. The term "meso" is not a proper word, because the mesentery and mesocolon contain all blood vessels and lymphatics with peritoneal attachment.

Description of Regional Lymph Nodes of the Pancreas and Lymph Node Metastasis

In the sixth edition by JPS, the lymph nodes related to the pancreas were classified into three groups: Groups 1, 2, and 3. Lymph node metastasis was described according to existence of metastasis in each group lymph nodes: N0 (no lymph node metastasis), N1 (lymph node metastasis in Group 1), N2 (lymph node metastasis in Group 2), and N3 (lymph node metastasis in Group 3). In the seventh edition, regardless of tumor location, the regional lymph nodes of the pancreas are defined as the following lymph node station numbers (Fig. 4) [12]: 5, 6, 7, 8a, 8p, 9, 10, 11p, 11d, 12a, 12b, 12p,13a, 13b,14p,14d, 17a, 17b, and 18 (Table 2). In case of metastasis in the other lymph node number (1, 2, 3, 4, 15, 16a1, 16a2, 16b1, 16b2, etc.), it is defined as M1.

The committee of JPS classification for seventh edition reevaluated the patient survival according to metastasis in the lymph node groups and the total numbers of lymph node metastasis using pancreatic cancer registry data by Japan Pancreas Society from 2001 to 2007 [13]. As a result, overall survivals between the patients with N2 and those with N3 were comparable and very poor, while there was significant difference between the patients with N0 and those with N1. According to the total numbers of lymph node metastasis, overall survival was significantly better in the patients with no lymph node metastasis followed by the patients with one to three lymph node metastases and those with four or more lymph node metastases in decreasing order (MST: 34.7, 21.9, 15.7 months, respectively) (Fig. 5). Given these results, the recording of lymph node metastasis in the seventh edition by JPS is shown as follows:

NX: Regional lymph nodes cannot be assessed.
N0: No regional lymph node metastasis.
N1: Regional lymph node metastasis.
N1a: Metastasis in one to three regional lymph nodes.
N1b: Metastasis in four or more regional lymph nodes.

Similarly, the AJCC proposed the change for N definitions in the eighth edition as follows: N0 = node negative, N1 = one to three nodes positive for metastatic disease, and N2 = four or more nodes positive for metastatic disease. The multi-institutional collected data analysis for all patients ($n = 1{,}551$) who underwent a R0 resection found that these two separate cutoffs were useful for stratification of prognosis [5].

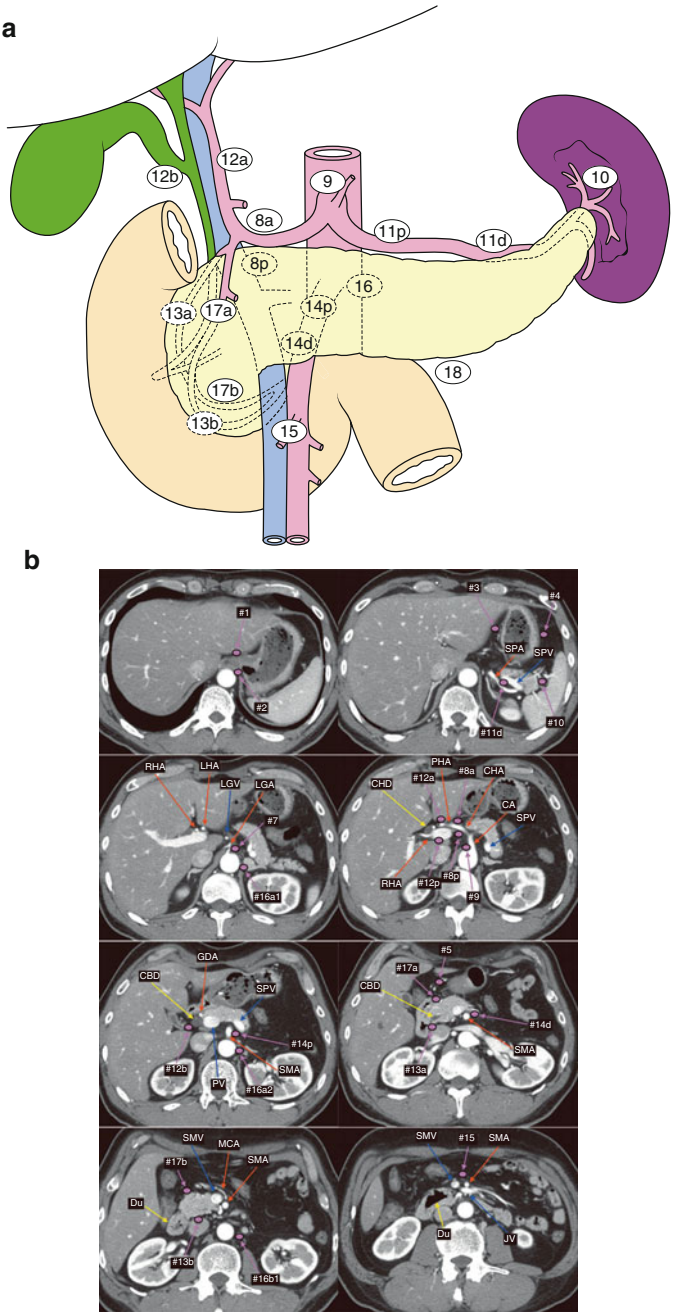


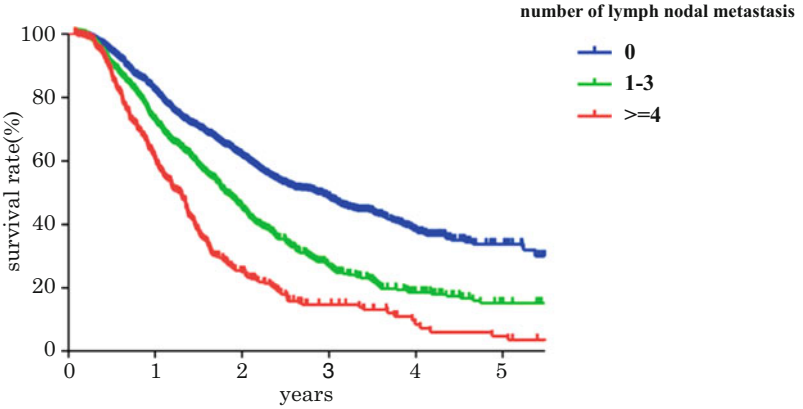
Fig. 4 Lymph node station numbers related to the pancreas in Japanese classification of pancreatic cancer, seventh edition (Kanehara & Co., Ltd., Tokyo, Japan). (**a**) Scheme of lymph node station numbers related to the pancreas. (**b**). MD-CT finding (cross section) showing peripancreatic lymph

Table 2 Numbers and names of lymph nodes related to the pancreas

Number	Name
1	Right cardial lymph nodes
2	Left cardial lymph nodes
3	Lymph nodes along the lesser curvature of the stomach
4	Lymph nodes along the greater curvature of the stomach
5[a]	Suprapyloric lymph nodes
6[a]	Infrapyloric lymph nodes
7[a]	Lymph nodes along the left gastric artery
8a[a]	Lymph nodes in the anterosuperior group along the common hepatic artery
8p[a]	Lymph nodes in the posterior group along the common hepatic artery
9[a]	Lymph nodes around the celiac axis
10[a]	Lymph nodes at the splenic hilum
11p[a]	Lymph nodes along the proximal splenic artery
11d[a]	Lymph nodes along the distal splenic artery
12a[a]	Lymph nodes along the hepatic artery
12p[a]	Lymph nodes along the portal vein
13a[a]	Lymph nodes on the posterior aspect of the superior portion of the head of the pancreas
13b[a]	Lymph nodes on the inferior aspect of the superior portion of the head of the pancreas
14p[a]	Lymph nodes along the proximal superior mesenteric artery
14d[a]	Lymph nodes along the distal superior mesenteric artery
15	Lymph nodes along the middle colic artery
16	Lymph nodes around the abdominal aorta
16a1	Lymph nodes around the aortic hiatus of the diaphragm
16a2	Lymph nodes around the abdominal aorta (from the superior margin of the celiac trunk to the inferior margin of the left renal vein
16b1	Lymph nodes around the abdominal aorta (from the inferior margin of the left renal vein to the superior margin of the inferior mesenteric artery)
16b2	Lymph nodes around the abdominal aorta (from the superior margin of the inferior mesenteric artery to the aortic bifurcation)
17a[a]	Lymph nodes on the anterior surface of the superior portion of the head of the pancreas
17b[a]	Lymph nodes on the anterior surface of the inferior portion of the head of the pancreas
18[a]	Lymph nodes along the inferior margin of the pancreas

[a]The regional lymph nodes of the pancreas

Fig. 4 (continued) node station numbers. *SPA* splenic artery, *SPV* splenic vein, *RHA* right hepatic artery, *LHA* left hepatic artery, *LGV* left gastric vein, *LGA* left gastric artery, *CHD* common hepatic duct, *CHA* common hepatic artery, *CA* celiac axis, *GDA* gastroduodenal artery, *CBD* common bile duct, *SMA* superior mesenteric artery, *MCA* middle colic artery



	MST (mo)	1-yr	2-yr	3-yr	5-yr	
0 (n=1003)	34.7	81.7%	61.3%	48.8%	33.8%	p<0.0001
1-3 (n=892)	21.9	72.8%	45.1%	26.8%	15.8%	
>=4 (n=409)	15.7	60.7%	25.0%	14.8%	4.9%	p=0.0001

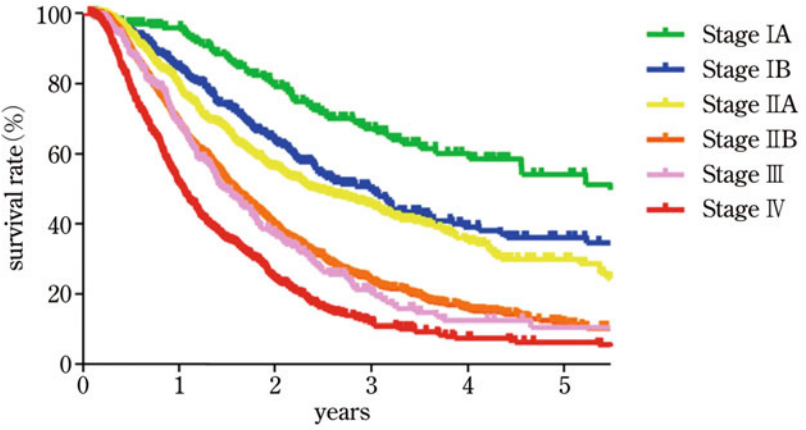
Fig. 5 Survival curves in the PDAC patients with resection according to the number of lymph node metastasis among regional lymph nodes of the pancreas (Kanehara & Co., Ltd., Tokyo, Japan). Pancreatic cancer registry data by Japan Pancreas Society 2001–2007 ($n = 2304$). *PDAC* pancreatic ductal adenocarcinoma

Table 3 Stage grouping in Japanese classification of pancreatic cancer seventh edition

Stage 0	Tis	N0	M0
Stage IA	T1 (T1a, T1b, T1c)	N0	M0
Stage IB	T2	N0	M0
Stage IIA	T3	N0	M0
Stage IIB	T1 (T1a, T1b, T1c), T2, T3	N1 (N1a, N1b)	M0
Stage III	T4	Any N	M0
Stage IV	Any T	Any N	M1

Stage Grouping

Stage grouping in the seventh edition by JPS is shown in Table 3. In the sixth edition, staging system was made based on data for resected cases of pancreatic cancer registry by JPS, focusing on stratification of prognosis according to each stage. In contrast, the JPS seventh edition basically adopted staging system of UICC seventh edition, focusing on enabling clinicians to decide treatment option for each stage. Roughly, stages I and II are initially resectable pancreatic cancer. Stage III is borderline resectable or locally advanced pancreatic cancer for which



	MST (mo)	1-yr	2-yr	3-yr	5-yr	
Stage IA (n=201)	69.4	95.9%	79.1%	67.1%	54.1%	
Stage IB (n=310)	36.1	84.7%	63.3%	50.0%	36.2%	p<0.0001
Stage IIA (n=615)	29.4	79.3%	56.0%	45.3%	29.9%	p=0.0757
Stage IIB (n=1367)	19.2	68.6%	39.2%	23.4%	11.8%	p<0.0001
Stage III (n=257)	18.1	68.4%	36.9%	20.4%	10.7%	p=0.4195
Stage IV (n=565)	12.7	51.5%	24.2%	12.2%	6.5%	p<0.0001

Fig. 6 Survival curves in the PDAC patients who underwent resection according to stage (Kanehara & Co., Ltd., Tokyo, Japan). Pancreatic cancer registry data by Japan Pancreas Society 2001–2007 ($n = 3315$). *PDAC* pancreatic ductal adenocarcinoma

neoadjuvant therapy may be recommended on the setting of clinical trial. Stage IV has distant metastasis for which systemic chemotherapy is recommended. When survival rates were retrospectively evaluated using pancreatic cancer registry data by JPS according to the current stage grouping, 5-year survival rates of stages IA, IB, IIA, IIB, III, and IV were 54.1, 36.2%, 29.9%, 11.8%, 10.7%, and 6.5% (Fig. 6). The significant difference of overall survival rates was found between IA and IB, IIA and IIB, and III and IV. In contrast, the survival rates were comparable between stages IIB and III: 11.8% versus 10.7% ($p = 0.4195$).

Classification of Resectability

Surgical resection is the only potentially curative therapy for long-term survival for pancreatic cancer. At the time of diagnosis of pancreatic cancer, however, only approximately 10–20% of patients are considered candidates for curative resection [14]. Therefore, it is important to define the resectability using common criteria from the perspective of determining treatment option and comparing outcomes. In the sixth edition of JPS and UICC seventh edition, there are no classification and criteria defining resectability.

The National Comprehensive Cancer Network (NCCN) has developed guidelines to define tumor resectability in pancreatic cancer based on MD-CT finding since 2006, in order to improve patient selection for surgery and to identify the likelihood of an R0 resection [15]. Using their criteria, pancreatic cancer is classified as resectable (R), borderline resectable (BR), locally unresectable (LUR), or metastatic. BR pancreatic cancer can be defined as one that increases the likelihood of an incomplete resection. On the contrary, LUR pancreatic cancer is locally advanced pancreatic cancer including tumors with SMA or CA encasement greater than 180° and unreconstructable portal vein (PV)/SMV occlusion. However, this guideline has been revised periodically and detailed, and this criteria focus on the final decision of resectability by only pancreatic surgeons. In the seventh edition by JPS, therefore, criteria defining resectability status based on the findings of dynamic CT have been established by thorough discussion among pancreatic surgeons, gastroenterologist, radiologist, and pathologist, taking NCCN guideline 2015 into consideration as follows:

Resectable: R

No tumor contact with the superior mesenteric vein (SMV) or portal vein (PV) or less than 180° contact or invasion without occlusion. Clear fat planes around the superior mesenteric artery (SMA), celiac axis (CA), and common hepatic artery (CHA), showing no contact or invasion

Borderline Resectable: BR

Subclassified according to SMV/PV invasion alone or arterial invasion

BR-PV (SMV/PV Invasion Alone)

No findings of contact and invasion of the SMA, CA, and CHA. Tumor contact or invasion of the SMV/PV of 180 or more degrees or occlusion of the SMV/PV, not exceeding the inferior border of the duodenum

BR-A (Arterial Invasion)

Tumor contact or invasion of the SMA and/or CA of less than 180° without showing stenosis or deformity. Tumor contact or invasion of the CHA without showing tumor contact or invasion of the proper hepatic artery (PHA) and/or CA

Unresectable: UR

Subclassified according to the status of distant metastasis

UR-LA (Locally Advanced)

Tumor contact or invasion of the SMV/PV of 180 or more degree or occlusion of the SMV/PV, exceeding the inferior border of the duodenum. Tumor contact or invasion of the SMA and/or CA of 180 or more degree. Tumor contact or invasion of the CHA showing tumor contact or invasion of the PHA and/or CA. Tumor contact or invasion of the aorta

UR-M (Tumor with Distant Metastasis)

Distant metastasis including non-regional lymph node metastasis.

BR pancreatic cancer is classified into the following two types according to the vascular invasion: BR-PV means the tumor whose vascular invasion is limited

within PV (portal vein) alone, and BR-A means the tumor with involvement of peripancreatic arteries such as SMA, CA, and hepatic artery (HA). This subclassification is based on the multicenter data collection by the Japanese Society of Pancreatic Surgery that BR-A increases the likelihood of an incomplete resection in comparison with BR-PV, showing significantly poor prognosis in the patients with BR-A [16, 17].

Resectability criteria of the seventh edition by JPS are considered to be utilizable for not only pancreatic surgeons but also gastroenterologist and radiologist, because they are objective criteria only based on dynamic CT findings by avoiding the subjective definitions such as "SMV/PV involvement allowing for safe and complete resection and vein reconstruction" in BR pancreatic cancer and "unreconstructible SMV/PV due to tumor involvement or occlusion" in UR pancreatic cancer [15]. Instead of these subjective definitions for SMV/PV involvement, the authors have adopted the objective definition: SMV/PV involvement exceeding or not exceeding the inferior border of the duodenum, as shown in Fig. 7. In the case of BR-PV in Fig. 7a, the tumor of pancreatic head has 180 or more degree contact/

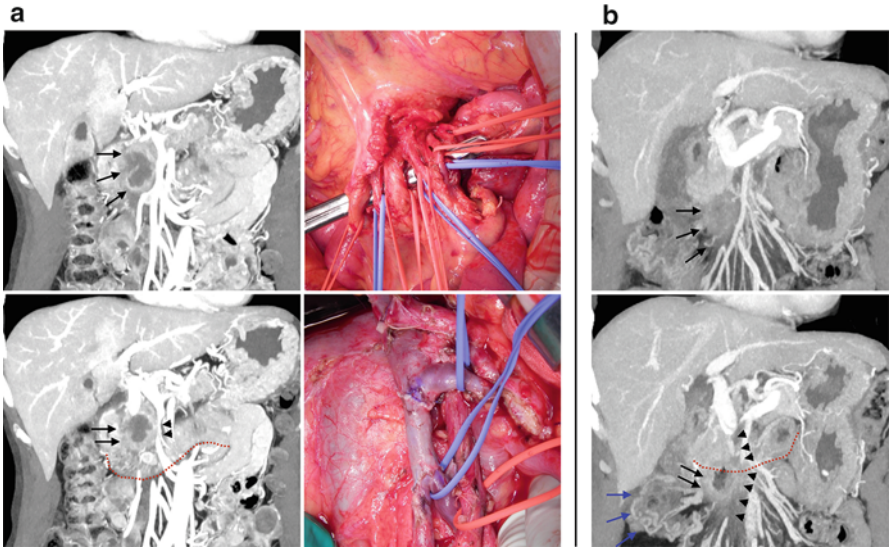


Fig. 7 Resectability criteria according to the degree of tumor invasion of the SMV/PV. (**a**) A case of BR-PV: the tumor of pancreatic head (*black arrows*) has 180 or more degrees of contact/invasion of the SMV/PV (*black arrow heads*), but not exceeding the inferior border of the duodenum (*right break line*). In this case, pancreaticoduodenectomy with combined resection of portal vein followed by reconstruction using the external iliac vein graft was performed after preoperative chemoradiotherapy in the Mie University School of Medicine, and negative surgical margin was confirmed (operative finding in middle pictures). (**b**) A case of UR-LA: the tumor of pancreatic head (*black arrows*) invades and occludes the PV/SMV, exceeding the inferior border of the duodenum (*right break line*). SMV/PV is completely occluded and collateral venous formation is found (*blue arrows*). This case was evaluated as unresectable even after chemoradiotherapy, and systemic chemotherapy was performed in the Mie University School of Medicine

invasion of the SMV/PV, but not exceeding the inferior border of the duodenum. In this case, pancreaticoduodenectomy with combined resection of portal vein followed by reconstruction using the external iliac vein graft was performed after preoperative chemoradiotherapy in the Mie University Hospital, and negative surgical margin was confirmed. In the case of UR-LA in Fig. 7b, the tumor of pancreatic head invades and occludes the PV/SMV, exceeding the inferior border of the duodenum. SMV/PV is completely occluded and collateral venous formation is found. This case was evaluated as unresectable even after chemoradiotherapy, and systemic chemotherapy was performed in the Mie University Hospital.

Criteria of Histological Response to Drug Therapy and/or Radiotherapy

Chemoradiotherapy and chemotherapy before surgery may provide for the early treatment of micrometastatic disease and allow for the identification of patients with metastatic disease and increase the R0 resection rate, resulting in a reduced risk for local recurrence and improvement in outcome. Especially for BR and UR-LA cases, systemic chemotherapy and chemoradiotherapy followed by curative-intent surgery have been widely adopted in recent years [14]. Given these background, it is required to establish uniformed criteria of histological response of drug therapy and/or radiotherapy for pancreatic cancer. The Evans grading system and the classification by the American Pathologists (CAP) grading protocol are the best studied scores [18, 19], and the relationship between histologic response and prognosis has been reported in recent years [20, 21]. The grading system of histological response in the seventh edition by JPS is shown as follows:

Grade 1: Poor or no response
Response to therapy is poor (estimated rate of residual tumor is 50% or more).
Grade 1a: estimated rate of residual tumor is 90% or more.
Grade 1b: estimated rate of residual tumor is 50% or more and less than 90%.

Grade 2: Moderate response
Cancer cells which are considered viable are moderately present (estimated rate of residual tumor is 10% or more and less than 50%).

Grade 3: Marked response
Cancer cells which are considered viable are few (estimated rate of residual tumor is less than 10%).

Grade 4: Complete response
No viable cancer cells are present.

*The estimated rate (%) of residual tumor is defined as the volume of cancer cells considered viable/the estimated tumor volume before treatment. As of host reaction to tumor destruction by preoperative therapy, xanthoglanulomatous change containing foamy histiocytes, pooling of mucin without cancer cells, infiltration of inflammatory cells, and fibrosis are important pathological features which enable us to estimate the tumor volume before treatment.

These criteria basically adopted the principle of both Evans grading system and CAP grading protocol, but they clearly describe how to estimate the rate of residual tumor cells. The estimated rate (%) of residual tumor is defined as the volume of cancer cells considered viable/the estimated tumor volume before treatment. As of host reaction to tumor destruction by preoperative therapy, xanthoglanulomatous change containing foamy histiocytes, pooling of mucin without cancer cells, infiltration of inflammatory cells, and fibrosis are important pathological features which enable us to estimate the tumor volume before treatment.

Conclusions

The revised seventh edition of JPS pancreatic cancer classification focuses on establishing consistency to UICC seventh edition, while originality of JPS classification is maintained as follows: anatomical definition of extrapancreatic nerve plexuses, N category based on numbers of lymph nodal metastasis among the regional lymph nodes, objective criteria of resectability only based on dynamic CT findings by avoiding the subjective definitions, and criteria of histological response to drug therapy and/or radiotherapy.

Cross-References

► Borderline Resectable Pancreatic Cancer
► Pancreatic Adenocarcinoma: CT and PET/CT

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Adjuvant Chemotherapy in Pancreatic Cancer

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Abstract

Pancreatic cancer is one of the major causes of cancer death. Most patients present with advanced disease, and only 10–15% of patients can undergo resection. Survival after curative surgery is poor, as recurrences occur either locally or distantly. Adjuvant therapy has been employed in large randomized trials to treat systemic disease and hopefully improve the poor prognosis. Chemoradiation, chemotherapy using 5-fluorouracil/folinic acid (5FU/FA), S-1, gemcitabine or gemcitabine plus capecitabine, and combination therapy have all been used in the adjuvant setting.

The results of the EORTC and ESPAC-1 trials have revealed that there is no survival advantage associated with adjuvant chemoradiation following resection for pancreatic cancer compared to no chemoradiation. There is no level 1 evidence, as yet that chemoradiation is superior to chemotherapy alone following surgery. Justification for the use of combination chemoradiation with follow-on chemotherapy is based on the results of an underpowered 1987 GITSG study, which closed prematurely and compared intervention to observation. The RTOG 9704 combination study did not demonstrate a survival difference between a 5FU-based regimen compared with a gemcitabine-based chemoradiation regimen. There is no completed randomized study comparing chemotherapy versus combination therapy.

There is a clear survival advantage with adjuvant 5FU/FA and single-agent gemcitabine based on the results from the ESPAC-1 and CONKO-001 study, respectively. The ESPAC-3 trial showed that these adjuvant regimens are equally effective, but gemcitabine has a better toxicity profile. In contrast, in a Japanese population, the JASPAC-01 trial demonstrated the superiority of S1 over gemcitabine. Adjuvant combination chemotherapy with gemcitabine plus capecitabine has been recently shown to provide a survival advantage compared with gemcitabine alone in Western patients in the ESPAC-4 trial. Phase III studies investigating other combination chemotherapy regimens are ongoing and will possibly increase the number of treatment options in this setting.

Keywords
Adjuvant chemotherapy · Meta-analysis · Pancreatic cancer · Randomized controlled trial

Introduction

The effective treatment of pancreatic adenocarcinoma is a huge challenge. Over the past three decades, there has been considerable progress toward understanding the biology of pancreatic cancer, refining imaging systems, improving surgical outcomes, and more recently focusing on new combination treatments and biomarkers to enable targeted therapies. The worldwide incidence is 337,872 cases per year resulting in 330,391 deaths, and in Europe pancreatic cancer accounts for 103,773 new cases and 104,481deaths each year [1]. In the USA in 2016, there were around 53,070 new cases of pancreatic cancer diagnosed with 41,780 deaths [2]. The incidence of pancreatic cancer has been rising, and it is likely to be the second leading cause of cancer deaths by 2030 [3]. The American Cancer Society's estimates for pancreatic cancer in the USA for 2017 are that about 53,670 people (27,970 men and 25,700 women) will be diagnosed with pancreatic cancer and that about 43,090 people (22,300 men and 20,790 women) will die of pancreatic cancer [4]. Pancreatic cancer accounts for about 3% of all cancers in the USA and about 7% of all cancer deaths. There has been some improvement in survival outcome with the 1-year survival rate of people with pancreatic cancer who do not have surgery rising to 29% and the 5-year survival rate rising to 7% [4, 5].

The outlook for those patients who can undergo surgical resection is better. In specialized centers, resection rates of above 15% can be achieved [5, 6]. Although surgery cannot guarantee a cure, the 5-year survival does improves to around 8–10% following resection [6, 7]. Naturally, there have been many attempts to improve survival by increasing the radicality of the surgical resection including by total pancreatectomy [8], multivisceral resections [9], and extended lymphadenectomy [10–12]. A meta-analysis has shown increased postoperative morbidity for extended lymphadenectomy over standard lymphadenectomy with pancreatectomy without any survival advantage, although there is trend favoring the more extended procedure [13].

The patterns of disease recurrence following resection include both locoregional failure and distant metastases [14]. Postmortem analyses have also shown that hepatic metastases are the direct cause of death due to metastatic disease in up to 80% of cases [15]. The use of adjuvant therapy is a logical strategy to target systemic disease and thereby improve survival.

Rationale for Adjuvant Chemotherapy

Pancreatic cancer is highly resistant to many standard chemotherapy regimens relative to other gastrointestinal cancers. This is a persistent problem and may be accounted for, in part, by the underlying tumor biology of pancreatic cancer. Few chemotherapeutic

agents have been shown to have reproducible response rates of more than 10%. 5FU is an inhibitor of thymidylate synthetase (essential for synthesis of DNA nucleotides) and has been the most widely used agent in advanced pancreatic cancer, with a median survival of around 5–6 months, and is better than the best supportive care [16, 17]. The nucleoside analogue, gemcitabine, replaced 5FU as the preferred drug in 1997 as the toxicity was relatively mild and achieved a better clinical response compared to 5FU (24% vs. 5%, respectively) [18]. Although the median survival improvement in favor of gemcitabine compared with 5FU was slight (5.7 vs. 4.4 months), the 1-year survival rate was more encouraging (18% vs. 2%) [18].

Combination chemotherapy has been developed to improve the outcomes observed with gemcitabine alone. Capecitabine is an oral, fluoropyrimidine carbamate that is sequentially converted to 5FU by three enzymes located in the liver and in tumors, including pancreatic cancer. Prospective studies including two randomized trials assessing the combination of capecitabine and gemcitabine have shown promise [19–21]. Meta-analysis of gemcitabine combination studies [22, 23] has demonstrated that combination gemcitabine chemotherapy results in significant survival benefit than gemcitabine alone (HR = 0.91; 95% CI, 0.85–0.97) and the best combinations may be with capecitabine or platinum-based agents, allowing for acceptable levels of toxicity of the combinations [24, 25].

Other combination chemotherapy regimens including 5FU plus folinic acid, irinotecan and oxaliplatin (FOLFIRINOX), and gemcitabine plus *nab*-paclitaxel have been shown to be superior to single-agent gemcitabine in terms of objective response rate (31.6% vs. 9.4% and 23% vs. 7%, respectively) and survival outcomes (median overall survival 11.1 vs. 6.8 and 8.5 vs. 6.7 months, respectively) and are now standard options for the first-line treatment of patients with good performance status [26, 27].

Novel biological agents against a variety of molecular targets have yet to have an impact in improving survival in pancreatic cancer including erlotinib, bevacizumab, aflibercept, axitinib, sorafenib and cetuximab [28]. The relative effectiveness of chemotherapy in patients with advanced pancreatic cancer led to the use of 5FU in the first wave of adjuvant chemotherapy trials. The emergence of gemcitabine as the standard for patients with advanced pancreatic cancer has influenced the next generation of adjuvant studies, while, more recently, combination chemotherapy regimens have been increasingly investigated in this setting.

Randomized Controlled Trials of Adjuvant Chemotherapy

The phase III randomized studies that have assessed adjuvant systemic chemotherapy in resected pancreatic cancer are summarized in Table 1.

Bakkevold et al.

This small multicenter study [29] from Norway was conducted between 1984 and 1987 and was the earliest study to compare chemotherapy to best supportive care

Table 1 Randomized controlled trials of adjuvant systemic chemotherapy

Series	Period	No. of patients	Regimen	Median survival (months)	Actuarial survival (%) 1 year	Actuarial survival (%) 2 years	Actuarial survival (%) 3 years	Actuarial survival (%) 5 years
Bakkevold et al. [29]	1984–1987	61	5FU/DOX/MMC	23	70	–	27	48
		31	–	11 ($p = 0.02$)	45	–	30	
Takada et al. [30]	1986–1992	81	MMC and 5FU	–	–	–	–	11.5
		77	–	–	–	–	–	18 NS
Kosuge et al. [31]	1992–2000	45	5FU and cisplatin	12.5	–	–	–	26.4
		44	–	15.8	–	–	–	14.9 ($p = 0.94$)
ESPAC-1 [32, 33] interim – all patients	1994–2000	238	5FU and FA	19.7	–	48.9	–	–
		253	–	14 ($p = 0.005$)	–	26.8	–	–
ESPAC-1 final – 2 × 2 factorial	1994–2000	149	5FU and FA	20.1	–	40	–	21
		143	–	15.5 ($p = 0.009$)	–	30.0	–	8.0
ESPAC-1 final – individual treatment groups	1994–2000	69	Observation	16.9	–	38.7	–	10.7
		75	5FU and FA	21.6	–	44.0	–	29.0
CONKO-001 [34]	1998–2004	189	Gemcitabine	22.1	–	–	34.0	22.0
		182	Observation	20.2	–	–	20.0	11.0 $p = 0.06$
CONKO-001 [35] longer follow-up	1998–2004	189	Gemcitabine	22.8	–	–	–	20.7
		182	Observation	20.2	–	–	–	10.4

(*continued*)

Table 1 (continued)

Series	Period	No. of patients	Regimen	Median survival (months)	Actuarial survival (%) 1 year	Actuarial survival (%) 2 years	Actuarial survival (%) 3 years	Actuarial survival (%) 5 years
JSAP-02 [36]	2002–2005	58	Gemcitabine	22.3	77.6	48.3	–	23.9
		60	Observation	18.4	75.0	40.0	–	10.6
ESPAC-3 [37]	2000–2007	551	5FU and FA	23.0	78.5	48.1	–	15.9
		537	Gemcitabine	23.6	80.1	49.1	–	17.5 NS
JASPAC-01 [38]	2007–2010	190	Gemcitabine	25.5	–	–	38.8	24.4
		187	S-1	46.5	–	–	59.7	44.1
CONKO-005 [40]	2008–2013	217	Gemcitabine	26.5	–	53.0	33.0	19.0
		219	Gemcitabine and erlotinib	24.6	–	54.0	36.0	28.0
ESPAC-4 [41]	2008–2014	366	Gemcitabine	25.5	–	–	–	16.3
		364	Gemcitabine and capecitabine	28.0	–	–	–	28.8 $p = 0.032$

5FU 5-fluorouracil, *FA* folinic acid, *DOX* doxorubicin, *MMC* mitomycin C, *NS* not significant

following resection. Sixty-one patients were randomized to receive either systemic chemotherapy with 5FU (500 mg/m^2), doxorubicin (40 mg/m^2), and mitomycin C (6 mg/m^2) (FAM) ($n = 30$) or observation ($n = 31$) following pancreatic resection. There were 47 patients with pancreatic ductal adenocarcinoma, and the rest had periampullary tumors. The FAM regimen was administered every 3 weeks for a total of six cycles. There was a statistically significant survival advantage for patients in the chemotherapy arm, who had a median survival of 23 months compared to the 11 months observed in the control group ($p = 0.04$), but this was lost at 5 years (4% vs. 8% $p = 0.10$). The poor long-term survival results have to be interpreted carefully due to the high initial drop-out rate (only 24 out of the original 30 patients randomized received chemotherapy) and appreciable toxicity associated with this regimen (only 13 patients completed all six scheduled courses). A further drawback of this study was that it pooled patients with pancreas and periampullary cancer, limiting the applicability of the results to pancreas cancer.

Takada et al.

Between April 1986 and June 1992, this multicenter Japanese trial [30] enrolled 508 patients who had undergone a resection for pancreatic, gall bladder, bile duct, or ampulla of Vater cancers. Patients were randomized to either the chemotherapy arm (mitomycin C 6 mg/m^2 and 5FU 310 mg/m^2 days 1–5 and days 15–20 followed by oral 5FU 100 mg/m^2 daily) or observation following surgery. Out of 173 patients with pancreatic cancer, 158 were eligible for survival analysis. The 5-year survival rate in patients with pancreatic carcinoma was 11.5% in the chemotherapy arm and 18.0% in the control arm, and this did not represent a significant difference. There was also no difference seen between the two treatment arms for the secondary endpoints of disease-free survival and time to recurrence. The poor performance of the chemotherapy regimen in this study could be attributed to the use of oral 5FU, which because of its hepatic metabolism has very poor efficacy compared to intravenously administered 5FU or specially designed oral fluoropyrimidines.

Kosuge et al.

A recent Japanese multicenter randomized controlled trial [31] evaluated chemotherapy with 5FU (500 mg/m^2) and cisplatin (80 mg/m^2) versus observation in 89 patients with pancreas cancer, recruited between 1992 and 2000. Enrolment was restricted to patients with microscopically clear resection margins (R0), and only two cycles of chemotherapy were administered. The authors concluded that there was no survival advantage for chemotherapy (median survival 12.5 months) compared to observation (median survival 15.8 months). Of interest is the 5-year survival figure, which was higher in the chemotherapy arm (26.4%) compared to the observation arm (14.9%) though this was not statistically significant ($p = 0.94$). The drawbacks of this study are that it was probably underpowered due to an

overambitious estimated survival difference and a suboptimal duration of the chemotherapy was used.

ESPAC-1

The European Study Group for Pancreatic Cancer Trial 1 (ESPAC-1) [32, 33] was a multicenter study, which used a 2 × 2 factorial design to assess the role of adjuvant chemotherapy or chemoradiation in pancreatic cancer. Following pancreatic resection, each patient was randomized to chemotherapy (bolus 5FU 425 mg/m^2 plus folinic acid 20 mg/m^2 days 1–5, monthly for six cycles) or chemoradiation (20 Gy dose to the tumor given in 10 daily fractions over a 2-week period plus an intravenous bolus of 5 FU 500 mg/m^2 each of the first three days of radiotherapy and again after a planned break of 2 weeks) or both treatments (i.e., chemoradiation followed by chemotherapy as above) or neither treatment (i.e., observation) . Randomization was stratified according to center and resection margin status. Between 1994 and 2000, a total of 289 patients were randomized into the 2 × 2 factorial design; a further 261 patients were randomized to either chemotherapy or chemoradiation versus observation outside the original design (ESPAC-1 plus). After a median of 47 months follow-up of patients in the 2 × 2 factorial design, the median survival was 20.1 months (95% CI, 16.5–22.7) among the 147 patients who received chemotherapy and 15.5 months (95% CI, 13.0–17.7) among the 142 patients who did not receive chemotherapy (hazard ratio for death, 0.71; 95% CI, 0.55–0.92; $p = 0.009$) (Fig. 1). Two-year and 5-year survival estimates were 40% and 21%, respectively, among patients who received chemotherapy and 30% and 8%, respectively, among patients who received no chemotherapy. Independently significant prognostic factors included tumor differentiation (HR1.89, 95% CI, 1.49–2.39), tumor size (HR1.21, 95% CI, 1.08–1.36), and positive lymph nodes (HR1.57, 95% CI, 1.18–2.09). Overall the influence of the type of surgery and the presence of complications on survival (in conjunction with clinicopathological variables) were studied using the Cox proportional hazard model. Postoperative complications or the type of resection did not impact on the survival benefit seen with adjuvant chemotherapy. The primary outcome of this study supports the use of 5FU/FA as standard adjuvant therapy in resected pancreatic cancer.

CONKO-001

This multicenter German study recruited 368 patients between July 1998 and December 2004. Following R0 or R1 pancreatic resection, 179 patients were randomized to receive gemcitabine (3 weekly infusions of gemcitabine 1,000 mg/m^2 given by intravenous infusion during a 30-min period, followed by a 1-week pause), and 177 patients were randomized to surgery alone. The primary endpoint of the trial was disease-free survival. There was a significant increase in median disease-free survival with gemcitabine (13.4 months 95% CI, 11.4–15.3) compared with control (6.9 months 95% CI, 6.1–7.8) but had just failed to demonstrate a significant advantage in median overall survival ($p = 0.06$) with gemcitabine compared with

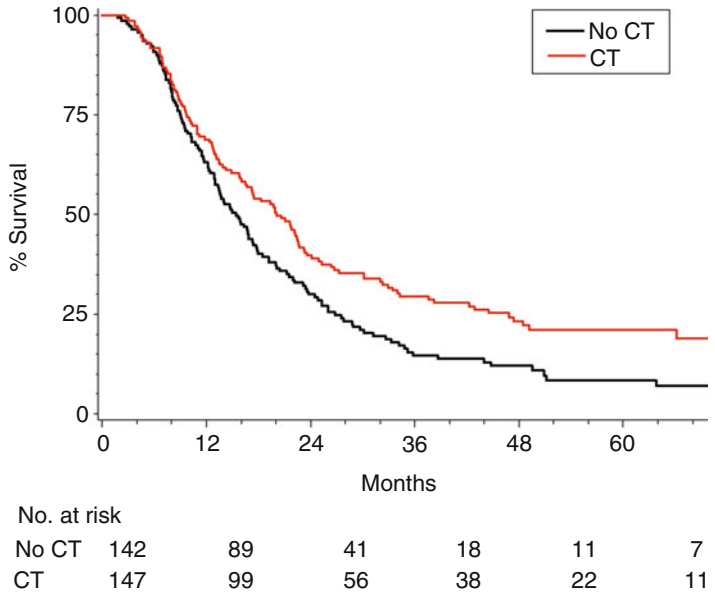

Fig. 1 Overall survival according to whether or not patients received chemotherapy (*CT*) in the ESPAC 1 trial final results, 2 × 2 factorial [32]

control [34]. However, the final analysis conducted after a median follow-up of 11 years showed also a significant advantage in overall survival [35]. Median disease-free survival for the gemcitabine group was 13.4 months compared to 6.9 months for the observation arm (HR 0.55, 95% CI, 0.44–0.69, $p < 0.001$). The estimated disease-free survival at 5 years was 16.6% in the gemcitabine group versus 7.0% in the observation group, respectively. There was a significant improvement in median overall survival with gemcitabine, 22.8 months, compared to observation alone, 20.2 months (HR 0.76, 95% CI, 0.61–0.95, $p = 0.01$). Estimated survival at 5 years was 20.7% for gemcitabine patients versus 10.4% for observation patients, respectively. The results of the CONKO-001 trial support the use of single-agent gemcitabine as an alternative option for the adjuvant treatment of pancreatic cancer.

JSAP-02

The JSAP-02 trial provided some evidence that the findings of the CONKO-001 trial could be generalized to Asian populations. This was a phase III study that was conducted in Japan and randomized macroscopically resected patients to adjuvant gemcitabine (3 weekly infusions of gemcitabine 1,000 mg/m^2 given by intravenous infusion, followed by a 1 week pause) or observation [36]. Of note, duration of treatment was shorter compared to the CONKO-001 trial with only three (instead of six) cycles of chemotherapy. The primary endpoint was overall survival and

118 eligible patients were recruited. Although a statistically significant improvement in disease-free survival was observed in favor of the chemotherapy arm (median disease-free survival 11.4 vs. 5.0 months; 2-year disease-free survival 27.2% vs. 16.7%; HR 0.60, 95% CI, 0.40–0.89, $p = 0.01$), no difference was found between arms in overall survival (median 22 overall survival 0.3 vs. 18.4 months; 5-year 23 overall survival 0.9% vs. 10.6%, HR 0.77, 95% CI, 0.51–1.14, $p = 0.19$). Given that the absolute advantage in favor of adjuvant gemcitabine in terms of both disease-free survival and overall survival appeared very similar to that reported by the investigators of the CONKO-001 trial, it is likely that the negative results of the JSAP-02 are due to the fact that the statistical design was too ambitious (i.e., target HR 0.55) and, therefore, the study underpowered to demonstrate a statistically significant difference in overall survival.

ESPAC-3

The ESPAC-3 trial was originally designed as a 3-arm, randomized, phase III trial to compare observation alone versus 24 weeks of adjuvant chemotherapy with either gemcitabine (3 weekly intravenous infusions of 1,000 mg/m^2, followed by a 1-week pause) or 5FU plus folinic acid (425 mg/m^2 and 20 mg/m^2, respectively, given as intravenous bolus on 5 consecutive days every 28 days) [37]. However, following the results of the CONKO-001 study, the observation arm was discontinued and the study continued as a 2-arm trial. The primary endpoint was overall survival. A total of 1,088 pancreatic cancer patients who had undergone a microscopically resection were enrolled. No statistically significant difference was observed between treatment arms, with median overall survival being 23.0 months (95% CI, 21.1–25.0) for patients treated with 5FU plus folinic acid and 23.6 months (95% CI, 21.4–26.4) for those randomly assigned to gemcitabine (HR 0.94, 95% CI, 0.81–1.08, $p = 0.39$). Overall survival at 2 years was 48.1% in the 5FU/folinic acid group versus 49.1% in the gemcitabine group (Fig. 2). Likewise, patients in both groups were reported to have similar outcome in terms of PFS (median and 2-year PFS 14.1 months and 30.7%, respectively, in the 5FU/folinic arm versus 14.3 months and 29.6%, respectively, in the gemcitabine arm, HR 0.96, 95% CI, 0.84–1.10, $p = 0.53$).

However, treatment with gemcitabine appeared to be better tolerated. A total of 14% of patients in the 5FU/folinic acid arm experienced a treatment-related serious adverse event compared with 7.5% in the gemcitabine arm ($p < 0.001$). Statistically significant differences in grade 3/4 toxicities between arms included leukopenia (10% vs. 6%) and thrombocytopenia (1.5% vs. 0%) which occurred more frequently in the gemcitabine group and stomatitis (10% vs. 0%) and diarrhea (13% vs. 2%) which were more common in the 5FU plus folinic acid group.

The efficacy findings of this study confirmed that gemcitabine and 5FU plus folinic acid are equally effective as adjuvant treatments for resected pancreatic cancer patients. However, the toxicity data support the contention that, in view of its more favorable safety profile, gemcitabine should be considered as the preferred therapy.

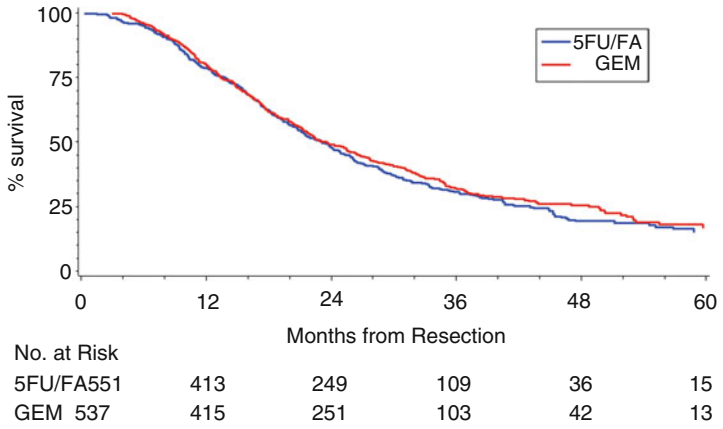

Fig. 2 Overall survival according to whether or not patients received adjuvant chemotherapy with 5-fluorouracil and folic acid (5FU FA) or gemcitabine (GEM) in the ESPAC-3 (v2) trial [37]

JASPAC-01

S-1 is an oral drug containing tegafur (a prodrug of the active compound 5FU), gimeracil (a dihydropyrimidine dehydrogenase (DPD) inhibitor), and oteracil potassium (an orotate phosphorribosyltransferase inhibitor). This combination was developed to increase the therapeutic ratio of 5FU by prolonging half-life and maintaining high levels of this agent in blood and tumor tissue while limiting the production of the same (and resulting toxicity) in the gastrointestinal tract. Further to the encouraging antitumor activity observed in studies conducted in the advanced setting, S-1 has been recently investigated as adjuvant treatment.

Japan Adjuvant Study Group of Pancreatic Cancer (JASPAC-01) was a randomized, open-label, phase III trial conducted in Japan [38]. In this study, patients who had undergone surgical resection (either R0 or R1) for stage I–III pancreatic ductal adenocarcinoma were randomized to receive standard gemcitabine (3 weekly iv infusions of gemcitabine 1,000 mg/m^2, followed by a 1-week pause) or S-1 (40–60 mg according to the BSA, twice daily for 28 days, followed by a 2-week pause) for 24 weeks. The primary endpoint was overall survival, and the study was powered to demonstrate non-inferiority of S-1. The per-protocol population included 377 patients. After a median follow-up >6 years, patients in the standard treatment arm had a median overall survival of 25.5 months and 5-year survival of 24.4% compared to 46.5 months and 44.1%, respectively, in patients who were assigned to the investigational arm. The HR for death of S-1 was 0.57 with the upper bound of the 95% CI (i.e., 0.72) being largely within the predefined non-inferiority margin (i.e., 1.25) (p value for non-inferiority <0.0001; p value for superiority <0.0001). A similar difference was observed between treatment arms with regard to relapse-free survival. Median relapse-free survival and 3-year relapse-free survival rate were 11.3 months and 22.6% in the gemcitabine group compared with 22.9 months and 39.2% in the S-1 group (HR 0.60, 95% CI 0.47–0.76, p <0.0001). Safety analysis

showed that gemcitabine treatment was associated with a statistically significantly increased risk of grade 3/4 leukopenia, neutropenia, and ALT/AST elevation, while patients randomized to S-1 experienced more grade 3/4 stomatitis and diarrhea.

The results of the JASPAC-01 trial suggest that S-1 should be the standard adjuvant treatment for pancreatic cancer in Japan and possibly in other Asian countries. Nevertheless, generalizability of these findings to Western populations is not recommended due to differences in terms of pharmacokinetic and safety of S-1 between Eastern and Western patients as previously reported in other studies.

CONKO-005

Based on the positive results of the NCIC CTG trial which demonstrated a small survival advantage for gemcitabine plus erlotinib compared to standard single-agent gemcitabine in the metastatic setting [39], the CONKO-005 trial investigated the same combination regimen as adjuvant treatment after microscopically radical (i.e., R0) resection [40]. In this phase III trial, patients were randomly assigned to receive 24 weeks of gemcitabine (3 weekly iv infusions of gemcitabine 1,000 mg/m^2, followed by a 1-week pause) or the same schedule of gemcitabine plus erlotinib (at a dose of 100 mg po, once daily). The primary endpoint was disease-free survival. A total of 436 patients were randomized. Results of this trial were presented at the 2015 ASCO Annual Meeting in Chicago, and no survival advantage from erlotinib was shown. Median disease-free survival was 11.6 months in both arms (HR 0.89, 95% CI 0.72–1.10). Also no significant difference in median overall survival was reported (24.6 months in the gemcitabine plus erlotinib arm vs 26.5 months in the gemcitabine alone arm, HR 0.90, 95% CI 0.71–1.15). Notably, a trend toward a better long-term outcome for patients who were treated with the combination treatment was observed (5-year overall survival rate 28% vs. 19%). In contrast to what was previously reported in the metastatic setting, a subgroup analysis did not confirm any correlation between grade of erlotinib-induced rash and outcome in the investigational treatment arm.

ESPAC-4

The ESPAC-4 trial was a randomized, open-label, phase III study comparing 24 weeks of standard single-agent gemcitabine (3 weekly iv infusions of gemcitabine 1,000 mg/m^2, followed by a 1-week pause) versus 24 weeks of combination chemotherapy with gemcitabine plus capecitabine (3 weekly iv infusions of gemcitabine 1,000 mg/m^2, followed by a 1-week pause and capecitabine 830 mg/m^2 twice daily orally for 21 days followed by 7 days of rest) as adjuvant treatment following macroscopically surgical resection [41]. The trial was conducted in Western countries including the UK, France, Germany, and Sweden. The primary endpoint was overall survival, and the study was powered to demonstrate a difference of 10% in a 2-year overall survival between treatment arms. A total of 722 patients were enrolled of whom 60% had undergone an R1 surgical resection

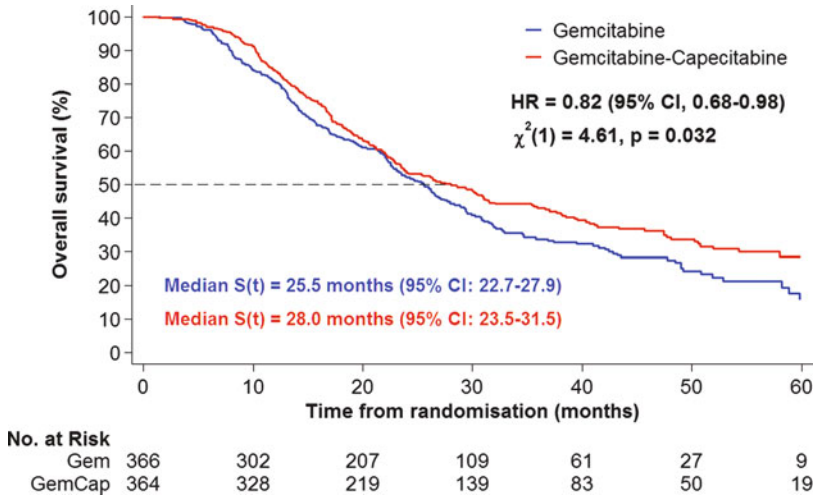

Fig. 3 Overall survival according to whether or not patients received adjuvant chemotherapy with gemcitabine (5FU FA) or gemcitabine plus capecitabine (GEM) in the ESPAC-4 trial [41]

and 80% had pathologically positive lymph nodes. The study was stopped prematurely by the Independent Data and Safety Monitoring Committee due to the positive results. Patients who were randomized to the combination treatment arm had a median overall survival of 28.0 months (95% CI 23.5–31.5) compared with 25.5 months (95% CI 22.7–27.9) for those who were treated with single-agent gemcitabine (HR 0.82, 95% CI 0.68–0.98, $p = 0.032$) (Fig. 3). At 5 years, 28.8% (95% CI 22.9–35.2) of patients in the combination therapy group were alive versus 16.3% (95% CI 10.2–23.7) in the control arm. Treatment with gemcitabine plus capecitabine was associated with an increased risk of grade ≥ 3 neutropenia (38% vs. 24%), hand-and-foot syndrome (7.0% vs. 0%), and diarrhea (5% vs. 2%), while more grade ≥ 3 infections/infestations were reported in the gemcitabine arm (7% vs. 3%). No difference in the proportion of patients experiencing a serious adverse event was observed between treatment groups (26% with gemcitabine and 24% with gemcitabine plus capecitabine).

The results of the ESPAC-4 trial set combination chemotherapy with gemcitabine plus capecitabine as a new standard of care for the adjuvant treatment of patients with curatively resected pancreatic cancer.

Predictive Biomarkers for Adjuvant Chemotherapy

There is no doubt that accurate patient selection is crucial to maximize the benefit from adjuvant chemotherapy. In this regard, studies have assessed the predictive or prognostic role of a number of tumor biomarkers in pancreatic cancer patients who are treated with curative surgical resection and adjuvant chemotherapy. Among all

biomarkers analyzed, the human equilibrative nucleoside transporter 1 (hENT1) appeared to be the most promising one. hENT1 is a membrane nucleoside transporter protein which is directly involved in the tumor cell uptake of gemcitabine. Therefore, it has been hypothesized that its expression levels could predict benefit from adjuvant gemcitabine. However, the results from retrospective analysis of randomized clinical trials have so far been contradictory.

The investigators of the ESPAC trials analyzed hENT1 expression (using the original Mackey mouse monoclonal anti-hENT1 antibody 10D7G2) in tissue microarrays from a total of 380 patients who were treated with adjuvant gemcitabine ($n = 176$), 5FU and folinic acid ($n = 176$), or observation alone ($n = 28$) within the context of the ESPAC-1 and ESPAC-3 studies [42]. They found that, among the group of gemcitabine-treated patients, those with high hENT1 expression had a significantly longer median overall survival (26.2 months) compared with patients who had low hENT1 expression (17.1 months) (HR 0.60, 95% CI 0.43–0.83, $p = 0.002$). In this group, hENT1 expression was an independent prognostic factor for overall survival ($P = 0.003$). In contrast, no difference in survival was observed according to the levels of hENT1 expression in the group of patients who received 5FU and folinic acid or no adjuvant chemotherapy.

A retrospective analysis of the CONKO-001 trial that included 156 patients (88 randomized to adjuvant gemcitabine and 68 to observation) found no association between hENT1 expression adjuvant gemcitabine and survival but used a completely different antibody (clone SP 120 rabbit antibody) [43].

Other potential candidates include dihydropyrimidine dehydrogenase, and thymidylate synthase metabolism involved in the metabolism of 5FU and carboxyl esterase-2 (CES2), which activates irinotecan into SN-38 which is part of the FOLFIRINOX regimen [28].

Future Studies

A further number of large multicenter randomized trials are actively recruiting. Addressing the question of whether combination chemotherapy regimens that have been recently shown to be superior to gemcitabine in the metastatic setting can have a role as postoperative treatments is the next logical step in the design of future adjuvant studies. In this regard, PRODIGE 24/ACCORD 24 and APACT are among the most interesting ongoing phase III studies, and the results are eagerly awaited.

PRODIGE 24/ACCORD 24 is a multicenter, randomized phase III trial investigating adjuvant treatment with a modified version of FOLFIRINOX (mFOLFIRINOX) [44]. Patients recruited in this study are randomized to standard gemcitabine (3 weekly iv infusions of 1,000 mg/m^2, followed by 1 week pause) or mFOLFIRINOX (2 weekly iv infusions of oxaliplatin 85 mg/m^2, irinotecan 150 mg/m^2, levogyre folinic acid 200 mg/m^2, and 46-h continuous infusion of 5FU 2,400 mg/m^2). The primary endpoint of the study is disease-free survival, and 490 patients are required.

APACT (ABI-007-PANC-003) is a multicenter, randomized phase III trial comparing gemcitabine plus nab-paclitaxel (3 weekly iv infusions of gemcitabine

1,000 mg/m^2 and paclitaxel 125 mg/m^2, followed by a 1-week pause) versus single-agent gemcitabine (3 weekly iv infusions of 1,000 mg/m^2, followed by a 1-week pause) [45]. Treatment duration is 24 weeks for both arms. The primary endpoint is disease-free survival, and 846 patients are required.

Rationale for Adjuvant Chemoradiation

We have robust evidence from completed large phase III trials that adjuvant chemotherapy should be used as standard for patients with resected pancreatic cancer. The argument to support the use of adjuvant chemoradiation is not quite so clear cut.

Radiation treatment after surgical resection of pancreatic cancer has been given with the idea of controlling any microscopic local residual disease. This is especially relevant considering that approximately 10–20% of resections are characterized by positive margins and recurrences following pancreaticoduodenectomy can occur at the site of resection. However, distant metastases are thought to be more common than local recurrences and account for the majority of tumor failures and cancer-related deaths in this setting.

External beam radiotherapy (EBRT) is delivered using chemosensitization (chemoradiation) usually with 5FU or gemcitabine. Although combining chemotherapy with radiation therapy can increase the risk of toxicities, chemoradiation has been shown to be superior to EBRT alone at least in the setting of locally advanced tumors [46]. Also, administering concurrent chemotherapy (despite at a low, chemoradiosensitizing dose) may potentially sterilize micrometastases and therefore reduce the risk of distant recurrence after surgical resection.

Nevertheless, compared with systemic chemotherapy, chemoradiation has been less investigated as adjuvant treatment for pancreatic cancer patients who had curative resection. Furthermore, clinical trials have so far reported contrasting results. These are reported in detail in the next paragraphs.

One of the issues associated with irradiation of the upper abdomen by external beam radiotherapy (EBRT) is the risk of considerable toxicity, especially gastrointestinal. Intraoperative radiation therapy (IORT) may be employed to reduce this by sparing normal tissues. The surrounding tissues can either be displaced or shielded, thereby allowing the delivery of larger radiotherapy doses in a single fraction to volumes harboring tumor cells. However, at the current time, there is no level 1 evidence to support its use in advanced pancreatic cancer. As most series on adjuvant IORT are dogged by small numbers, inclusion of all stages of the disease, heterogenous treatment strategies, and retrospective design, it is difficult to draw conclusions or make recommendations on IORT [47]. The one small randomized trial on IORT [48] was published in abstract form and found no difference in survival between surgery only and IORT (median survival 12 months in both groups).

Intensity-modulated radiotherapy (IMRT) could provide another option to minimize the risk of radiation treatment-related toxicities [49]. This has been increasingly

investigated in pancreatic cancer and is being used in large randomized clinical trials of adjuvant chemoradiation.

Randomized Controlled Trials of Adjuvant Chemoradiation

EORTC 40891

The role of postoperative chemoradiotherapy has been assessed in two large randomized studies (Table 2). In a multicenter prospective randomized trial, Klinkenbijl et al. [50] recruited 218 patients with either pancreatic head (stage pT1–2, pN0-1a) or periampullary cancer (stage pT1–3, pN0-1a). Randomization was to observation or radiotherapy with split course radiotherapy (40 Gy) and concurrent 5FU as continuous infusion. Median survival in the overall study population was 19 months in the observation group and 24.5 months for the treatment group (log rank $P = 0.208$). In patients with pancreatic cancer, the trend was in favor of chemoradiation, with the median overall survival being 12.6 months in the observation group and 17.1 months in the treatment group ($p = 0.099$). A subsequent report [51] on the long-term survival of patients from this trial, after a median follow-up of 11.7 years, reaffirmed that there was no difference in overall survival between the two arms (death rate ratio 0.91, 95% confidence interval 0.68–1.23; $p = 0.54$). The overall 10-year survival was 18% in the entire population and 8% in the subgroup of pancreas head cancers. The patterns of recurrent disease observed in both arms of the trial were very similar, and in each case over 70% of patients had distant metastases. These findings, again, highlight the need for a systemic component when considering adjuvant therapy for pancreatic cancer. The limitations of this study can be identified as a lack of maintenance chemotherapy and a questionable statistical design that limited its ability to detect a benefit for adjuvant chemoradiation.

ESPAC-1

As previously described, the ESPAC-1 study [32, 33] was an international multicenter randomized trial that originally used a two-by-two factorial design allocation, to address the issues of adjuvant chemotherapy and adjuvant chemoradiation in patients with resected pancreatic cancer ($n = 289$). The 2×2 factorial designs create four arms, namely, observation, chemoradiotherapy alone, chemotherapy alone, and chemoradiotherapy, followed by chemotherapy but only two permissible statistical comparisons, namely, chemotherapy versus no chemotherapy and chemoradiotherapy versus no chemoradiotherapy. Patients who were randomized to chemoradiotherapy received a dose of 40 Gy (20 Gy in 10 fractions, repeated after a 2-week pause) plus 5FU (500 mg/m^2 as a bolus infusion on the first 3 days of each cycle of radiotherapy). In the two-by-two factorial design, 145 patients were randomized to the chemoradiotherapy arm (72 of which received chemoradiotherapy followed by 5FU-based chemotherapy), while 144 were randomly assigned to no

Table 2 Adjuvant chemoradiotherapy: Randomized controlled trials

Series	Period	Number of patients	Regimen	Median survival (months)	Actuarial survival (%) 1 year	Actuarial Survival (%) 2 years	Actuarial Survival (%) 3 years	Actuarial Survival (%) 5 years
Klinkenbijl et al. [50]	1987–1995	110	40 Gy + 5FU	24.5	41	–	–	10
		108	–	19 ($p = 0.208$)	51	–	–	20
ESPAC 1- [32, 33] interim - all patients	1994–2000	175	40 Gy + 5FU	15.5	–	24.6	–	–
		178	No 40Gy + 5FU	16.1 ($P = 0.235$)	–	23.5	–	–
ESPAC-1 final – 2 × 2 factorial	1994–2000	145	40 Gy + 5FU	15.9	–	29	–	10
		144	No 40Gy + 5FU	14.8 ($p = 0.05$)	–	41	–	20
ESPAC-1 final - individual treatment groups	1994–2000	69	Observation	16.9	–	38.7	–	10.7
		73	40 Gy + 5FU	13.9	–	21.7	–	7.3

5FU fluorouracil, *FA* folinic acid, *Gem* gemcitabine, *CRT* chemoradiation

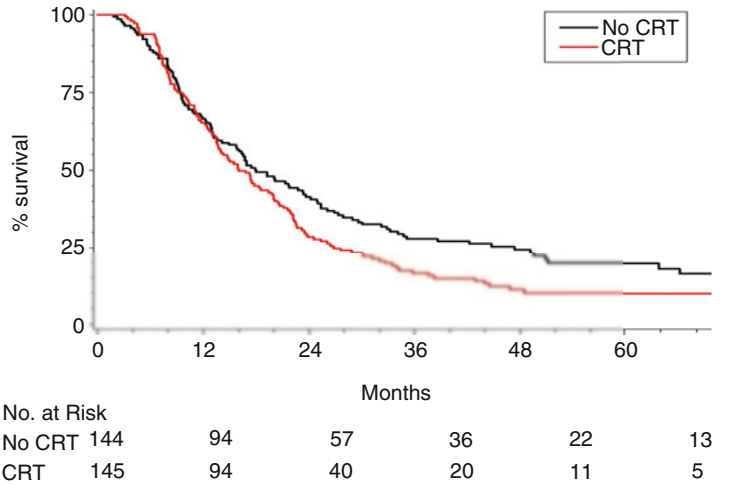

Fig. 4 Overall survival according to whether or not patients received adjuvant chemoradiotherapy (*CRT*) in the ESPAC 1 trial final results, 2 × 2 factorial [32]. *CT* chemotherapy

chemoradiotherapy (75 received 5FU-based chemotherapy and 69 observation alone). In the final analysis, the median survival was 15.9 months in the chemoradiotherapy arm and 17.9 months in the group who were not assigned to receive chemoradiotherapy (HR 1.28, 95% CI 0.99–1.66, $P = 0.05$) (Fig. 4). The estimated 5-year survival was 10% in the chemoradiotherapy arm compared to 20% in those who did not receive chemoradiotherapy ($p = 0.05$). The lack of a survival advantage following chemoradiotherapy could be due to delays in administering radiation in patients who suffered postoperative complications. This reduces the potential benefit of chemotherapy that is derived by administering it as soon as possible after resection. The arguments that the radiation given during the ESPAC1 trial was substandard or not exposed to rigorous quality control do not stand up, given that the survival in the individual groups is the same or superior to that observed in North American randomized studies and was bases on an intention to treat analysis including those who did have any chemoradiation.

The lack of convincing data from these phase III studies emphasizes the problems encountered when trying to justify the use of adjuvant chemoradiation for these patients. The possibility that in fact chemoradiation may have a negative impact on survival cannot be ignored. At the present time, the use of adjuvant chemoradiation cannot be recommended as standard therapy.

Rationale for Adjuvant Combination Therapy

The relative failure of chemoradiation to significantly improve survival following pancreatic resection led to the hypothesis that adjuvant chemoradiotherapy and follow-on chemotherapy should be a more successful approach. Evidence, however,

from meta-analysis of studies in advanced cancer has shown that there is no survival difference between chemoradiotherapy plus follow-on chemotherapy and chemotherapy alone [46]. Phase III studies have provided further evidence of the effect of chemoradiotherapy and follow-on chemotherapy in advanced pancreatic cancer; however, the message is mixed. For example, a phase III study compared chemoradiotherapy and follow-on gemcitabine with gemcitabine alone in patients with locally advanced disease [52]. The results were not encouraging as the combination therapy was more toxic and less effective than gemcitabine alone. Another randomized phase III study of 74 patients with locally advanced pancreatic cancer again compared gemcitabine versus chemoradiotherapy and follow-on gemcitabine. This study also found increased toxicity with the combination, but it was manageable. The median survival time, however, increased in the combination group versus the gemcitabine alone group (9.2 months 95% CI 7.8, 11.4 vs. 11.0 months 95% CI 8.4, 15.5 respectively). The 95% confidence intervals overlapped indicating that the difference was not statically different using a two-sided p test although the authors reported a significant difference using a one-sided p test [53].

Randomized Controlled Trials of Adjuvant Combination Therapy

GITSG 9173

The Gastrointestinal Tumor Study Group (GITSG) trial 9173 sets the trend for the use of chemoradiotherapy followed by chemotherapy in resectable disease [54, 55]. This trial randomized 43 patients either to observation or to receive combined treatment (chemoradiotherapy followed by chemotherapy) in the form of split course EBRT (40 Gy) and concurrent 5FU, followed by 5FU for 2 years (Table 3). The study was terminated prematurely both because of a low rate of accrual and because of an increasingly large difference in survival between the study arms. The median survival for the adjuvant treatment group was 20 months, significantly longer than the 11 months in the no adjuvant treatment arm. Actuarial survival rates at 2 years were 43% (95% CI 25–63) and 18% (95% CI 8–36), respectively. Because there were so few cases, a further 30 patients were registered (not randomized) to the treatment arm, and the median survival in this group was 18 months, with a 2-year survival rate of 46%. Owing to the small number of patients, the 95% confidence intervals of the survival curves were so large as to overlap with survival curves in patients receiving no additional treatment. Thus no convincing conclusion could be derived from this study, though it must be noted that the benefit from treatment could have been due to the maintenance chemotherapy used in this study.

RTOG 9704

The Radiation Therapy Oncology Group Study 9704 [56], a phase III trial, compared pre- and post-chemoradiation gemcitabine (at a dose of 1,000 mg/m^2/day) to pre-

Table 3 Adjuvant chemoradiotherapy and follow-on chemotherapy (combination therapy): Randomized controlled trials

Series	Period	Number of patients	Regimen	Median survival (months)	Actuarial survival (%) 1 year	Actuarial Survival (%) 2 years	Actuarial Survival (%) 3 years	Actuarial Survival (%) 5 years
GITSG 9173 [54, 55]	1987–1995	21	40 Gy + 5FU, with 5FU maintenance	21	–	43	–	19
		22	–	10.9 ($p = 0.03$)	–	18	–	5
ESPAC-1 [32, 33] final – individual treatment groups	1994–2000	69	Observation	16.9	–	38.7	–	10.7
		72	40 Gy + 5FU, with 5FU/FA maintenance	19.9	–	35.5	–	13.2
RTOG [56] 9704 – all patients = 538, eligible = 442	1998–2002	221	Gem pre-CRT, 50.4 Gy + 5FU, gem post CRT	18	–	–	–	–
		221	5FU pre-CRT, 50.4 Gy + 5FU, 5FU post CRT	16 ($p = 0.15$)	–	–	–	–
Head of pancreas only eligible = 381		187	Gem pre-CRT, 50.4 Gy + 5FU, gem post-CRT	20.6	–	40	32	–
		194	5FU pre-CRT, 50.4 Gy + 5FU, 5FU post-CRT	16.9 ($p = 0.033$)	–	35	21	–
IFN α-2b [62]	Not reported	57	5FUFA	28.5	–	–	–	–
		53	50.4 Gy + Cisplatin/5FU/ IFN α-2b followed by continuous 5FU	32.1	–	–	–	–

5FU 5fluorouracil, *Gem* gemcitabine, *CRT* chemoradiotherapy

and post-chemoradiation 5FU (at a dose of 250 mg/m^2/day given as a continuous infusion). Both arms of the study received 5FU-based chemoradiation (50.4 Gy), with the chemotherapy given for 3 weeks pre- and 12 weeks post-chemoradiation. Over 4 years, 538 patients were recruited, exceeding the planned target of 330 patients. Patients were stratified by size of the tumor, involvement of lymph nodes, and surgical resection margin. Analysis was restricted to the 451 eligible patients. Treatment groups were well balanced with the only exception of T stage, as more patients in the gemcitabine arm had T3/4 tumors compared to the 5FU arm (81% vs. 70%, $p = 0.01$). There was no difference in overall survival between the two arms (log rank $p = 0.34$). On the other hand, the rate of grade 3/4 adverse events was significantly higher in the gemcitabine arm (79% vs. 62%, $p < 0.001$), this being largely due to a significant difference in hematological toxicity (58% vs. 9%; $p < 0.001$). Of note, a subgroup analysis of the 388 patients with pancreas head cancer revealed a better survival for the gemcitabine group (i.e., median overall survival 20.5 vs. 16.9 months), and this was statistically significant after adjusting for prognostic factors (HR = 0.80; 95% CI = 0.63–1.00; $p = 0.05$). Comparison with the individual groups in the ESPAC 1 trial suggests better survival times associated with chemotherapy alone when compared with the combination group (and better survival overall), although the trial was not designed to look at this specific question (see Tables 1, 2, and 3). Good-quality data are lacking to support the use of adjuvant chemoradiation for pancreatic cancer [57]

It is now increasingly important to incorporate translational research into large prospective adjuvant studies to identify prognostic and predictive biomarkers and to better understand the underlying mechanisms of action. Two prognostic studies based on data and tissue from this trial have been completed. The RTOG 9704 study identified that the post-resection CA19.9 level was a significant predictor of overall survival [58] and that hENT1 expression of the tumor tissue using immunohistochemistry (with the original Mackey mouse monoclonal anti-hENT1 antibody 10D7G2) was an independent prognostic factor in the gemcitabine group [59].

Interferon-Based Chemoradiation

Based on the chemo- and radiosensitizing properties of interferon (IFN) as well as its ability to modulate the immune system, several phase II adjuvant studies have used IFN-based protocols in the adjuvant setting of pancreatic cancer and reported interesting results with median overall survival times of 25–27 months [60, 61]. More recently, a large randomized phase III trial compared chemoradiation with 5FU (200 mg/m^2/day, continuous infusion), cisplatin (30 mg/m^2, once a week), and IFN α-2b (3 million units, three times a week) plus EBRT (50.4 Gy) followed by two cycles of continuous 5FU versus chemotherapy with bolus 5FU and folinic acid (425 and 20 mg/m^2, respectively, on 5 consecutive days every 28 days for 6 cycles) in patients who had undergone microscopically resection for pancreatic adenocarcinoma [62]. The primary endpoint was overall survival. A total of 132 patients were randomized, while the per-protocol population consisted of 110 patients. No

difference in overall survival was found in both the intention-to-treat and per-protocol population. In the former, median survival was 26.5 months in the investigational arm compared to 28.5 months in the standard arm (HR 1.04, 95% CI 0.66–1.53, $P = 0.99$), while in the latter these figures were 32.1 months and 28.5 months, respectively ($P = 0$ 0.49). Of note, IFN-based chemoradiation was found to be significantly toxic with 85% of patients experiencing grade 3/4 adverse events compared to 16% of patients in the chemotherapy alone arm. Furthermore, investigational therapy was also associated with deterioration of a number of QoL parameters. Based on these results, the investigators themselves did not recommend further investigation of IFN-based therapies in this setting.

Future Studies

The results of the clinical trials that have been conducted so far suggest that adjuvant chemotherapy should be considered as the standard treatment for pancreatic cancer patients who undergo macroscopically radical surgical resection, regardless of the status of the surgical margins (i.e., either R0 or R1). On the other hand, the currently available data do not support the routine use of chemoradiotherapy.

One of the main concerns regarding the use of concurrent chemoradiotherapy in the adjuvant setting of pancreatic cancer is the natural course of this disease which is characterized by a high risk of postoperative metastatic dissemination. However, it is unknown whether administering chemoradiotherapy after chemotherapy may possibly confer a survival advantage.

The EORTC/FFCD/GERCOR 40013/22012/0304 phase II study randomized patients who had undergone surgical resection receive either four cycles of gemcitabine 1,000 mg/m^2 over 30 min weekly for 3 weeks and then 1-week rest (control arm) or gemcitabine 1,000 mg/m^2 for two cycles followed by weekly gemcitabine 300 mg/m^2 with concurrent radiation of 50.4 Gy given in 28 fractions of 1.8 Gy (experimental arm) [63]. This regimen was found to be feasible and well tolerated. However, no difference in both disease-free survival and overall survival was observed between treatment arms.

It is possible that the negative results are secondary to the relatively early switch to chemoradiation in the investigational arm. On the other hand, delivering chemoradiation after a reasonably long period of systemic control with adjuvant chemotherapy may allow selection of patients who are more likely to benefit from further locoregional treatment. This treatment strategy is being assessed in the randomized phase II/III clinical trial RTOG 0848/EORTC-40084-22084 [64]. In this study, patients who are disease-free after five cycles of adjuvant chemotherapy with gemcitabine plus or minus erlotinib are randomized to receive either one more cycle of adjuvant chemotherapy or the same followed by sequential fluoropyrimidine-based (i.e., 5FU or capecitabine) chemoradiotherapy (50.4 Gy). The primary endpoint of the study is overall survival, and 950 patients are estimated to be required.

Novel treatment approaches in the adjuvant setting include the investigation of immunomodulatory agents in combination with standard chemotherapy and

chemoradiotherapy. Targeting the immunosuppressive microenvironment of pancreatic cancer, a well-established contributor to the biological aggressiveness and inherent treatment resistance of this disease, is the rationale behind the use of these novel strategies. A number of agents are currently under investigation in this setting including immune checkpoint inhibitors and vaccine therapies, but these have proved to be disappointing.

The results of a phase III trial using a whole cell vaccine, the IMmunotherapy for Pancreatic RESectable cancer Study (IMPRESS), were negative [65]. This randomized phase III trial ($n = 722$) compared adjuvant chemotherapy with gemcitabine alone or in combination with 5FU-based chemoradiotherapy plus or minus algenpantucel-L, a whole cell vaccine consisting of HAPa-1 and HAPa-2, two human pancreatic cancer cell lines. The median overall survival was 30.4 months in the control arm compared with 27.3 months in the investigational arm. In the same groups, 3-year overall survival was 41.4% and 42.1%, respectively.

A combination using GVAX pancreas, based on a pancreatic cell line modified to secrete granulocyte-macrophage colony-stimulating factor (GM-CSF), an immune-stimulatory cytokine, and CRS-207 which is attenuated *Listeria monocytogenes* expressing mesothelin, another immune-stimulatory molecule had poorer survival than CRS-207 alone or chemotherapy [66]. This phase IIb ECLIPSE trial in the third line and greater setting reported a median overall survival of 3.8 months for patients treated with the GVAX pancreas and CRS-207 combination, 5.4 months for patients treated with CRS-207 alone, and 4.6 months for patients given chemotherapy.

A phase II trial evaluating necuparanib (M402), a heparinoid with antitumor activity, in combination with nab-paclitaxel and gemcitabine in patients with advanced metastatic pancreatic cancer was discontinued after an interim futility analysis of 57 deaths from 120 randomized patients showed disappointing efficacy [67].

Demcizumab (OMP-21 M18) is a humanized monoclonal antibody directed against the N-terminal epitope of Notch ligand delta-like 4 (DLL4) that binds to the membrane-binding portion of DLL4 and prevents its interaction with Notch-1 and Notch-4 receptors that mediate angiogenesis. The randomized phase II YOSEMITE trial in first-line pancreatic cancer patients with metastatic disease randomized 207 patients to nab-paclitaxel, gemcitabine plus placebo, or to nab-paclitaxel, gemcitabine plus one 70-day truncated course of demcizumab, or to nab-paclitaxel, gemcitabine plus two 70-day truncated courses of demcizumab. The trial did not meet the primary endpoint of progression-free survival, and at the interim median analysis, overall survival was 13.2 months in the pooled demcizumab arms, but had not been reached in the control arm at the time of these analyses [68].

The Role of Adjuvant Regional Therapy

Adjuvant intra-arterial chemotherapy and chemoradiation strategies have been evaluated in a number of historical studies. More recently, Hayashibe et al. treated nine patients with coeliac artery infusion of cisplatin (CDDP) and 5FU following

pancreatic resection with a median overall survival of 15.8 months [69]. Another study in non-randomized setting gave adjuvant intra-arterial chemotherapy 5-fluoruracil 750 mg/m^2, leucovorin75 mg/m^2, epirubicin 45 mg/m^2, and carboplatin 225 mg/m^2 (FLEC regimen) every 3 weeks for three cycles alone ($n = 24$) or with follow-on systemic gemcitabine ($n = 23$) after resection for pancreatic cancer [70]. The overall median disease-free survival was 18 months, and median overall survival was 29.7 months [70].

In a randomized controlled study, patients who had undergone resection for pancreatic or periampullary cancer were randomized to receive either intra-arterial mitoxantrone, 5FU, leucovorin, and cisplatin in combination with 30 × 1.8 Gy radiotherapy ($n = 59$) or no adjuvant treatment ($n = 61$) [71]. There was no significant effect on local recurrence, the development of liver metastases, and overall survival [71].

Meta-Analyses

There have been several meta-analyses of adjuvant therapy in pancreatic cancer. Composite data from the ESPAC-1 and ESPAC-3(v1) trials has confirmed a significant survival advantage of adjuvant 5-fluorouracil and folinic acid compared to observation for pancreatic cancer [72]. Stocken et al. performed a meta-analysis using individual patient data from four ($n = 875$) out of the five selected randomized controlled trials (total = 939) [73]. Assessment of adjuvant chemotherapy trials revealed a 25% reduction in the risk of death (hazard ratio = 0.75, CI: 0.64, 0.90, $P_{strat} = 0.001$) with chemotherapy compared to the no chemotherapy arm. On the other hand, there was no significant difference between chemoradiation versus no chemoradiation (hazard ratio = 1.09, 95% CI: 0.89, 1.32, $P_{strat} = 0.43$). In both the comparisons assessed, there was significant intertrial heterogeneity. On subgroup analysis, chemoradiation was more effective ($\chi^2 = 4.2$, $P = 0.04$) and chemotherapy less effective ($\chi^2 = 7.3$, $P = 0.007$) in patients with positive resection margin. These results provide strong evidence for institution of adjuvant systemic chemotherapy following curative surgery. Another more recent meta-analysis concentrated on adjuvant 5FU-based chemoradiotherapy for resectable pancreatic adenocarcinoma and found only limited benefit for adjuvant chemoradiation, essentially reinforcing the findings of Stocken et al. [74].

A meta-analysis following the publication of the CONKO-001 included five trials, with 482 patients allocated to the chemotherapy group and 469 patients to the control group [75]. Four studies were used to assess median survival which demonstrated a significant advantage for chemotherapy over control. Five studies were used to assess 5-year survival, and there was no significant difference between the chemotherapy and control, but the drawbacks include the lack of individual patient data, the omission of results from the ESPAC-1 plus patients [33], and the longer-term follow-up of the CONKO-001 trial [35] and of course could not include the very recent results of JASPAC-1 and ESPAC-4 [38, 41].

More recently, a meta-analysis of nine randomized clinical trials including 3033 patients has analyzed survival benefit and safety data associated with 6 different management options: observation, chemotherapy with 5FU, chemotherapy with gemcitabine, 5FU-based chemoradiotherapy, chemoradiotherapy plus 5FU chemotherapy, and chemoradiotherapy plus gemcitabine chemotherapy [76]. Final results showed that, compared to observation, adjuvant chemotherapy with either 5FU or gemcitabine (HR 0.62, 95% credible interval 0.42–0.88 and 0.68, 95% credible interval 0.44–1.07, respectively) and chemoradiation plus either 5FU chemotherapy or gemcitabine chemotherapy (HR 0.54, 95% credible interval 0.15–1.80 and 0.44, 95% credible interval 0.10–1.81, respectively) were associated with better overall survival. Risk reduction was statistically significant only for 5FU chemotherapy. In contrast, no improvement in overall survival was found with chemoradiotherapy (HR 0.91, 95% credible interval 0.55–1.46). The use of chemoradiation plus chemotherapy appeared to provide only a slight survival advantage compared to chemotherapy, and this was even less evident when the HRs were adjusted taking into account the proportion of patients with positive lymph nodes. Finally, an increased risk of grade 3/4 toxicities was reported with chemoradiation plus chemotherapy, and this was especially true for hematological adverse events during chemoradiation plus gemcitabine chemotherapy.

Conclusion

This is an important and encouraging time for pancreatic cancer, there are data from large randomized adjuvant studies which have been completed, further trials are under development, and further studies are currently active. This situation is a vast improvement from that a decade ago. There is a general shift in the thinking about pancreatic cancer and its treatment. Although there has been a deep divide in the approach to pancreas cancer management between Europe and America, with the former adopting adjuvant chemotherapy, and the latter continuing to promote chemoradiation with follow-on chemotherapy, these studies have contributed to a change in attitudes, such that a more common approach using systemic chemotherapy alone is evolving.

A better understanding of the biology of pancreatic cancer indicates that this is a systemic disease very early in its pathogenesis reflecting the need for systemic (chemo) therapy and also suggesting the need to explore its use in the neoadjuvant setting for resectable disease [5, 77]. This needs to be balanced however with the prospect that, at least in some cases, neoadjuvant therapy may select and promote more aggressive cancer cell clones. Thus timely surgery with adjuvant therapy must remain at the center of our logical analysis in taking this and other novel concepts forward.

Analysis of the ESPAC-3 data has shown that the most effective approach to adjuvant chemotherapy is to deliver all six cycles of chemotherapy in the adjuvant setting [78]. Survival is not influenced by whether adjuvant chemotherapy is started before eight weeks of surgery or between 8 and 12 weeks after surgery. The issue

seems to hinge on fatigue: start the chemotherapy too soon when the patient still has postoperative fatigue; then it becomes very difficult to give all six cycles. On the one hand, waiting longer for the patient to reach full recovery with little or no fatigue than most patients will be able to continue through to all six cycles.

With improving standards in surgery, the outlook in terms of survival is improving for patients with borderline resectable and locally advanced pancreatic cancer [79], and resectability is increasing with the introduction of neoadjuvant chemotherapy, most notably FOLFIRINOX [80].

More research is needed to understand the potentially negative survival effect of radiotherapy on pancreatic cancer. A recent study in genetically engineered (KPC) mice and mice with orthotopic tumor cell transplants from KPC mice tumors showed that radiation produced a higher frequency of advanced pancreatic intraepithelial lesions [81]. There were more foci of invasive cancer than pancreata of unexposed mice (controls), and radiation reduced survival time by more than 6 months. Radiation-treated mice had tumors with a higher proportion of immune-suppressive M2-like macrophages, fewer CD8(+) T cells, and greater CD4(+) T cells of T-helper 2 and T-regulatory cell phenotypes than controls. Moreover it was shown that adoptive transfer of T cells from irradiated cancers to tumors of control mice accelerated tumor growth. Radiation induced production of MCSF by the cancer cells, while a neutralizing antibody against MCSF prevented the radiation-induced tumor promoting macrophages and increased the antitumor T-cell response and slowed tumor growth [81].

There is now exceptionally good level 1 evidence for adjuvant chemotherapy in pancreatic cancer following resection, as demonstrated by two large randomized controlled trials and supported by the results of meta-analyses. Single-agent chemotherapy with either gemcitabine or 5FU plus folinic acid (with the former being preferred over the latter due to its better safety profile) has been universally accepted as a routine treatment approach based on the results of the CONKO-001 and ESPAC-1, ESPAC-3, and ESPAC-4 trials and in Japan, S-1 based on the JASPAC-1 trial.

The key to the future of adjuvant therapy in pancreatic cancer will be the identification of novel and effective agents and better biomarker technology underpinned by translational research which will inform the design of future trials. Ultimately this will ensure that patients will be able to receive selective therapy to achieve the most benefit. Finally, it is worth noting that the modest survival outcome currently achievable with the available adjuvant therapies has led to an increased interest in the investigation of neoadjuvant treatment strategies in patients who have upfront resectable or borderline resectable tumors. If these novel approaches are demonstrated to provide a better long-term tumor control, then a reappraisal of the role of adjuvant therapy within the new therapeutic algorithm of early-stage pancreatic cancer will be necessary.

The findings from the recently completed ESPAC-4 trial indicate that combination chemotherapy with gemcitabine plus capecitabine is superior to single-agent gemcitabine and therefore should be adopted as new standard of care in this setting in Western countries and is mirrored in national and international guidelines including those of the American Society of Clinical Oncology [82].

Key Practice Points

- Adjuvant gemcitabine and 5FU-based chemotherapy significantly improve survival compared to observation.
- Give all six cycles of chemotherapy after surgery.
- The start of chemotherapy can be delayed until the patient has little or no fatigue after surgery; this may be up to 12 weeks.
- Gemcitabine and 5FU-based chemotherapy do not produce different survival rates as adjuvant treatments, but the safety profile of gemcitabine is better than that of 5FU-based chemotherapy.
- Adjuvant combination chemotherapy with gemcitabine plus capecitabine significantly improves survival compared to single-agent gemcitabine.
- Adjuvant chemotherapy with S-1 significantly improves survival compared to single-agent gemcitabine in Japanese patients.
- Adjuvant chemoradiation has not been shown to improve survival
- Adjuvant chemoradiation after adjuvant chemotherapy may not offer improved survival compared to chemotherapy alone – trial results are still awaited from the RTOG 0848 trial [64].

Future Research Directions

- Improved predictive biomarkers
- Improved combination chemotherapies
- Neoadjuvant plus adjuvant therapy
- Integrated translational research
- Development of biological therapies
- Development of immunotherapies
- Improved prognostic biomarkers
- Standardized pathological assessment

Cross-References

- ▶ Adjuvant Chemoradiation Therapy for Pancreatic Cancer
- ▶ Arterial Resection in Pancreatic Cancer
- ▶ Circulating Tumor Cells
- ▶ Clinical Decision-Making in Pancreatic Cancer
- ▶ Controversies in Pathology Reporting and Staging
- ▶ Evolution of Pancreatic Cancer Surgery
- ▶ Neoadjuvant Chemotherapy in Pancreatic Cancer
- ▶ Staging and Postoperative Outcomes using the International Study Group of Pancreatic Surgery (ISGPS) Classifications

► Surgical Resection for Pancreatic Cancer using the International Study Group of Pancreatic Surgery (ISGPS) Classifications
► Venous Resection in Pancreatic Cancer Surgery

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Useful Websites

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http://www.pancreaticcancer.org.uk/index.htm
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http://www.corecharity.org.uk/Pancreatic-Cancer.html
http://www.lctu.org.uk/aboutus/default.asp

Adjuvant Chemoradiation Therapy for Pancreatic Cancer

Adeel Kaiser, William F. Regine, Naimish Pandya, and Michael C. Garofalo

Contents

Abstract

Despite newer treatment modalities, overall outcome for pancreatic cancer remains poor and has changed very little during the past 30 years. Surgery remains the mainstay, but delivery of adjuvant postoperative therapy has been shown to be essential for long-term survival. Large, prospective randomized studies have revealed conflicting data on whether chemotherapy alone or combination chemoradiation is optimal. They have also triggered debates regarding the sequencing of adjuvant therapy strategies. Unfortunately, marked discrepancies exist with patient selection as well as trial design among these studies, resulting in inadequate comparisons of their conclusions. Nevertheless, like other gastrointestinal malignancies, it appears that adjuvant combination chemoradiation is superior to chemotherapy alone when the data is critically analyzed. This critical

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examination of the published data to date is provided in the forthcoming chapter, along with an assessment of what is needed for future trials to determine the optimal adjuvant treatment modality and improve overall outcome for pancreatic cancer patients.

Keywords
Radiotherapy · Radiation · Adjuvant radiation · Adjuvant radiotherapy · Adjuvant chemoradiation · Pancreatic cancer · Cancer of the pancreas · Algenpantucel-L · SMAD4

Introduction

It is estimated that more than 53,000 Americans will be diagnosed with cancer of the pancreas in 2016 [1] . The incidence of pancreatic cancer continues to rise on an annual basis. This is matched by an equally horrifying and also increasing estimated death rate. In 2016, it is anticipated that more than 41,000 deaths will occur as a result of this cancer. Long-term survival continues to remain very dismal and is less than 5%. Despite modern therapies, overall outcome has changed very little during the past quarter century. Surgery continues to remain the key therapeutic intervention for improved long-term outcome; however, only 10–20% of newly diagnosed pancreatic cancer patients are even surgical candidates [2]. Therefore, it is imperative to widen the number of durable survivors from this small pool of resectable patients. Experiments with newer therapeutics and altered treatment strategies are ongoing to accomplish this. The use of adjuvant therapy after surgery is becoming more established to improve long-term outcome. However, numerous randomized clinical trials in this realm have provided conflicting results regarding the role of chemotherapy versus chemoradiotherapy in the adjuvant setting. After decades of using 5-flurouracil (5-FU), gemcitabine has established superiority. Enhancement in the delivery of the radiation therapy is minimizing toxicity and also contributing to improved overall outcome. The ideal time sequence of combined adjuvant therapies is also an area of active deliberation. This chapter will review the historical trials that have defined the potential benefits for adjuvant chemoradiation and examine the evidence on hand that establish its advantage over adjuvant chemotherapy alone.

Rationale for Adjuvant Chemoradiation in Pancreatic Cancer

Though only a small percentage of patients are eligible to undergo pancreatoduodenctomy, an even smaller percentage of pancreatic cancer patients go on to have a definitive cure from this surgery. Unfortunately, even a pathological R0 resection will not guarantee long-term survival, as many patients eventually fail and ultimately die of disease progression [3]. Available data indicate only a 27.8 month median survival when margin negative surgery is completed [4]. Furthermore, up to 75% of

these recurrent pancreatic cancer patients will have a component of local failure after their resection and about 25% will be local failures only [5]. The high rate of both locoregional and distant recurrences among pancreatic cancer patients following surgery is the impetus to developing aggressive adjuvant treatment strategies and improving overall survival [6–8].

Pancreatic ductal carcinomas frequently harbor genetic alterations that have been shown to predict for local failure. These mutations are being studied now as a mean to stratify patients who would benefit most from local therapy. In general, many pancreatic cancers harbor KRAS, CDKN2A/p16, TP53, and SMAD4/DPC mutations [9, 10]. Of these, SMAD4, a tumor suppressor gene, is inactivated in 53–67% of cases.

Though the role of adjuvant therapy is more established today, the type of adjuvant treatment strategy continues to remain in controversy and evolve, while continued investigations into combinations of chemotherapy, radiation therapy, and biologic therapy are ongoing [11]. The retroperitoneal location of the pancreas and its proximity to major neurovascular structures make resections with wide negative margins challenging, and often result in close or microscopically positive surgical margins (R1 resection). As demonstrated in other gastrointestinal malignancies, residual microscopic locoregional disease can be eradicated by adjuvant chemoradiation. The locoregional control benefits conveyed by adjuvant chemoradiation have been proven in phase III esophageal, stomach, and rectal cancer trials, which have subsequently translated into improved overall survival [12, 13]. During the past two decades, various randomized prospective clinical trials have attempted to determine the optimal adjuvant therapy, and though the debate still rages, it is becoming clearer that proper patient selection remains the key to achieving the most favorable long-term outcome. Initial investigations among inoperable locally advanced pancreatic cancers revealed the benefit of the use of chemoradiotherapy [14, 15]. This led to three randomized trials that incorporated this strategy among operable cases [16–18]. However, competing with this notion, two other randomized European trials espouse that radiation may not be crucial in the adjuvant setting [19, 20]. Nevertheless, data from recent trials support the use of radiation, but optimal patient selection remains imperative.

Randomized Prospective Trials

GITSG Trial

The GITSG trial [16] was the first of such trials that randomized pancreatic cancer patients to chemoradiation or observation among those who had undergone a potentially curative (R0) resection with negative surgical margins. Treatment included a split-course of 40 Gy of external beam radiation therapy (EBRT) delivered over 6 weeks. The treatment delivered the first 20 Gy of EBRT over 2 weeks with concurrent bolus 5-FU chemotherapy (500 mg/m^2) during the first 3 days. This was repeated again during weeks 5 and 6 after a 2-week break. This was then followed by weekly bolus 5-FU given as maintenance chemotherapy for 2 years or

until disease progression. Although the trial was slow to accrue and a marked difference in survival led to its early closing, a 43 patient analysis revealed a statistically significant doubling in median overall survival and modest improvement in 5-year survival for patients receiving adjuvant split-course chemoradiation. The 21 patients randomized to adjuvant split-course chemoradiation had a median survival, 2-year survival, and 5-year survival of 21 months, 43%, and 19% compared to 11 months, 18%, and 5%, respectively, for the observation group ($p = 0.03$). There were no long-term life threatening complications or deaths attributable to therapy and only 2 of the 51 total treated patients (4%) in the GITSG study developed late treatment-related complications [21]. However, the predominant critiques of this trial include its limited power, inadequate quality assurance of radiation delivered and the inability to complete the maintenance chemotherapy treatment by a significant number of its patients. To compensate for the small patient population and verify its results, the GITSG treated a nonrandomized cohort of 32 patients similar to the adjuvant chemoradiotherapy treatment arm of its original trial. This cohort achieved similar results, with median, 2-year, and 5-year survivals of 18 months, 46%, and 17%, respectively [16]. These additional results further validated the benefit seen with adjuvant chemoradiation and led to the adoption of adjuvant chemoradiation as the standard of care in the United States.

EORTC Trial

The European Organization for Research and Treatment of Cancer (EORTC) conducted an analogous multicenter study to evaluate the potential benefit of adjuvant chemoradiation by randomizing resected patients to chemoradiation or observation [15, 17]. Unlike the GITSG trial, the EORTC treated both pancreatic and periampullary adenocarcinoma and allowed both R0 and R1 surgical resections. Of the 207 patients, 103 were randomized to observation and 104 to the split-course chemoradiotherapy regimen similar to that used in the GITSG study. However, their chemotherapy consisted of continuous infusion of 5-FU (25 mg/kg/day) instead of the bolus dosing concurrently with the radiation, and also did not include maintenance 2-year chemotherapy after completing the chemoradiotherapy treatment. The results revealed a statistically insignificant lack of improvement in median overall survival, 2-year survival, or 5-year survival, with 24.5 months, 51%, and 28% for the treatment arm, compared with 19 months, 41%, and 22% in the observation group, respectively. A sub-analysis of only pancreatic head cancer revealed the median duration of overall survival and 2-year and 5-year survivals to be 17.1 months, 37% and 20% within the treatment arm compared to 12.6 months, 23%, and 10% for the observation arm, which were also not statistically significant. Though the authors questioned the utility of chemoradiotherapy as adjuvant treatment based on their results, a reanalysis using a one-sided log-rank test demonstrated a 14% survival difference from the observation, which was statistically significant ($p = 0.049$) [22].

The discrepancy between the EORTC and GITSG trial results can likely be explained by its different patient population and modified treatment algorithm. Approximately 20% of the enrolled patients had an R1 resection, which are known to have a poorer prognosis [23]. Whereas a little less than 50% of the enrollees were

found to have periampullary adenocarcinoma, which portend a better long-term survival [24]. The EORTC study did not include systemic maintenance 5-FU chemotherapy after completion of combination therapy, though systemic 5-FU has not demonstrated significant survival benefit among pancreatic cancer patients. Furthermore, 20% of its patients did not receive any adjuvant therapy among the treatment arm and up to 44% had not received the intended chemotherapy. When the analysis was limited to those patients with only pancreatic head adenocarcinoma, the results of the treatment arm were slightly inferior to the GITSG trial. Finally, both studies were under-powered to offer any definitive results.

ESPAC-1 Trial

The European Study Group for Pancreatic Cancer (ESPAC) conducted the prospective multicenter ESPAC-1 trial in Europe in an attempt to clarify the need for radiation in adjuvant therapy in resected pancreatic cancer [19, 25]. The trial enrolled 541 patients with pancreatic adenocarcinoma only, who underwent a potentially curative resection, irrespective of the margin status. Patients within this trial underwent a double randomization of chemotherapy versus chemoradiotherapy and yes versus no to each option using a 2 × 2 factorial design that created four groups: (a) observation, (b) chemotherapy alone, (c) chemoradiotherapy, and (d) chemoradiotherapy followed by maintenance chemotherapy. The chemoradiotherapy used in two of the arms of the study modeled the GITSG and EORTC split-course therapy of 40 Gy over 6 weeks with concomitant 5-FU chemotherapy (500 mg/m^2) on days 1–3 of weeks 1 and 5. The chemotherapy used in one arm and the maintenance chemotherapy that followed chemoradiotherapy in another arm was modeled after the Mayo regimen, using daily bolus 5-FU (425 mg/m^2) for the first five consecutive days in a 28-day cycle for a total of six cycles. The design and the statistical analysis of the study were highly complex, but intended to compare no chemotherapy (groups a + c above) versus chemotherapy (groups b + d) and chemoradiotherapy (groups c + d) versus no chemoradiotherapy (groups a + b). After a median follow-up of 47 months, the estimated 5-year survival of patients randomized to chemoradiotherapy was 10% versus 20% for no chemoradiotherapy. The patients who received chemotherapy had a significantly higher 5-year survival when compared with those who did not receive chemotherapy (21% vs. 8%). Detailed results of this complicated trial can be found within Table 1.

Based on the results of this trial, the authors of ESPAC-1 concluded that adjuvant chemotherapy was beneficial and chemoradiotherapy detrimental to overall survival among resected pancreatic cancer patients. However, though this had been a bold effort to define adjuvant therapy, the trial suffered from numerous shortcomings [31]. First, it endured a complex trial design that not only led to creating inadequately powered four separate groups, but the combination analysis among the groups did not allow for a clear delineation of the effects of chemotherapy alone or chemoradiotherapy alone. Additionally, the clinicians were allowed to administer chemotherapy or chemoradiotherapy ("backdrop therapy") prior to enrolling the patient into the trial. This clearly confounded end results as not only does it create a selection bias by the clinician but the "background therapy" confounds the effects

Table 1 Results of randomized multicenter Phase III and nonrandomized adjuvant trials

Study	n	Treatment schema	R0 resection (%)	Median Survival (mo)	Overall survival (%) 2-yr	3-yr	5-yr
Randomized studies							
GITSG [16]	21	CRT (split course XRT)	100	21	43		19
	22	Observation	100	9	18		5
	32	CRT (split course XRT)	100	18	46		17
EORTC [17]	104	CRT (split course XRT)	81	25	51		28
	103	Observation	75	19	41		22
Subanalysis: pancreatic head	55	CRT (split course XRT)		17	37		20
	57	Observation		13	23		10
RTOG 9704 [18] – analysis: pancreatic head only	187	Gem + CRT (continuous)	39	21		31	
	201	5-FU + CRT (continuous)	44	17		22	
CONKO-001 [20]	179	Chemotherapy only (gemcitabine)	81	23		37	21
	175	Observation	85	20		20	9
ESPAC-1 [25, 26]							
	69	(a) Observation		17			11
	75	(b) Chemotherapy alone		22			29
	73	(c) Chemoradiotherapy (split course XRT)		14			7
	72	(d) CRT (split course) + maintenance chemo		20			13
Combined analysis							
	147	Chemotherapy (groups b + d)	81	20	40		21
	142	No chemotherapy (groups a + c)	84	16	30		8
	145	Chemoradiotherapy (groups c + d)	81	16	29		10
	144	No chemoradiotherapy (groups a + b)	84	18	41		20
Nonrandomized studies							
Johns-Hopkins and Mayo clinic collaboration [27]	583	Chemoradiotherapy (5-FU-based chemo)	69	21	45		22
	509	Observation	65	16	35		16
ACOSOG Z05031 [28]	89	Chemoradiotherapy (5-FU + Cis + IFNα + XRT)	75	27	55		

(*continued*)

Table 1 (continued)

Study	n	Treatment schema	R0 resection (%)	Median Survival (mo)	Overall survival (%) 2-yr	3-yr	5-yr
Mehta VK et al. (Stanford University Med Ctr) [29]	52	Chemoradiotherapy (concurrent 5-FU only)	65	32	62	39	
Reni M et al. (Milan, Italy) [30]	51	Chemoradiotherapy (PEGF chemo)	74	27	53		22

CRT chemoradiotherapy (5-FU + RT), *XRT* radiation therapy, *Gem* gemcitabine, 5-FU 5-flurouracil, *Cis* cisplatin, *IFNα* interferon-α, *PEGF* cisplatin + epirubicin + gemcitabine + 5-flurouracil, *mo* month, *yr* year

of the investigative treatment. Finally, no quality control of radiation treatment was performed, leading to under dosing and heterogeneous radiation treatment fields. Also, the time interval to begin adjuvant therapy markedly varied between arms, again leading to selection bias of delayed treatment for poorer performance status patients. The specific reasons for the inferior radiation results in ESPAC-1 are unclear, but the fact that the median overall survival (16.9 months) for observation arm was much better than that seen in the GITSG (10.9 months) or EORTC (12.6 months) trials also remains unexplained. Thus, the unique results of ESPAC-1 need further validation before any conclusions can be drawn regarding the role of chemotherapy or chemoradiation in the adjuvant setting.

RTOG 97-04 Trial

The Radiation Therapy Oncology Group (RTOG) led a collaborative cooperative group effort in further delineating the optimal adjuvant treatment for resected pancreatic cancer patients [18]. This study was intended to evaluate the addition and determine the superior chemotherapy treatment to the optimal dose of 50.4 Gy of radiation with concurrent 5-FU continuous infusion. This study also included R0 and R1 resected patients, but only allowed pancreatic adenocarcinoma histology. After surgical resection, patients received one cycle of chemotherapy, then chemoradiation, followed by three additional cycles of chemotherapy. The chemotherapy cycles consisted of either gemcitabine (1,000 mg/m^2) given once weekly for 3 weeks with 1 week off or continuous infusion 5-FU (250 mg/m^2/day) for 3 weeks followed by a week off. In both arms, EBRT was delivered continuously for a total dose of 50.4 Gy combined with concomitant 5-FU (250 mg/m^2/day) delivered by continuous infusion throughout the duration of the radiation treatment. Following an initial dose of 45 Gy, a final 5.4 Gy was delivered to a "boost" field of the tumor bed as defined by the preoperative tumor volume. A total of 451 randomized surgically

resected patients were analyzed, of which 230 were randomly assigned to the 5-FU-based regimen and 221 were assigned to the gemcitabine arm. Patients were stratified according to surgical margins (R0 vs. R1 vs. Unknown), tumor diameter ($<$3 cm vs. $\geq$3 cm), and nodal status (N0 vs. N1). Results of pancreatic head cancers revealed a median and 3-year overall survival for patients treated with gemcitabine-based chemoradiotherapy to be 20.5 months and 31% versus 16.9 months and 22%, respectively, for the 5-FU-based arm, which were not statistically significant. When adjusting for surgical resection status, tumor diameter, and nodal status, a statistically significant difference was observed favoring the gemcitabine arm. Based on these results, the authors concluded that gemcitabine was superior to 5-FU when added to chemoradiation, and that future adjuvant chemoradiotherapy trials should build upon a gemcitabine-based chemoradiotherapy backbone.

One of the key differences of the RTOG trial and its predecessors was the utility of central quality assurance of the radiation treatment. Prior studies were found to have an unacceptable protocol variance which led to inequity in the treatment delivered. The impact of RT quality assurance and compliance among RTOG 97-04 patients showed a statistically increased ($p = 0.0077$) median survival for those patients undergoing radiation per protocol (1.74 years) versus substandard radiation (1.46 years) [32]. In fact, the quality of radiation correlated more strongly with survival than the assigned treatment artm ($p = 0.014$). Also, when comparing the 5-FU arm of the RTOG trial with the GITSG and EORTC trials, the median OS was inferior. This is likely accounted by the greater number of patients with a R1 or unknown surgical resection margin, which are known to have poorer prognosis. Nevertheless, this trial and the CONKO-001 (discussed below) substantiate the use of gemcitabine-based chemotherapy in the adjuvant setting ahead of 5-FU. Finally, since no chemotherapy alone arm was included in this trial, no definite conclusions could be drawn from this trial about the role of chemotherapy alone as adjuvant therapy.

CONKO-001 Trial

The Charité Onkologie group conducted a large European multi-institutional prospectively randomized trial that addressed the question of only adjuvant chemotherapy and no radiation. No chemoradiation was utilized in this trial. Three hundred sixty-eight resected pancreatic adenocarcinoma patients were enrolled and stratified among the treatment and observation arms. Only surgically resected patients with known margins were allowed to enter the trial. Also, patients with CA 19-9 greater than 2.5 times the upper limit of normal were excluded from the trial. Randomization occurred into two arms, one received six cycles of systemic chemotherapy given once weekly for 3 weeks of gemcitabine (1,000 mg/m^2) followed by 1 week off or the other which was an observation arm with no adjuvant therapy. Contrary to the other randomized trials, the CONKO-001 investigators powered their study to determine a difference in the disease free interval between the two arms. At a median follow-up of 136 months, the median disease free survival was 13.4 months in the adjuvant treatment group versus 6.7 months in the observation group. Median overall survival was also found to statistically improve with chemotherapy. Median

overall survival and 5-year and 10-year survival were 22.8 months, 20.7% and 12.2% versus 20.2 months, 10.4% and 7.7%, respectively, for the observation arm [33]. When stratified by subpopulations of Node negative versus Node positive, both disease free survival and overall survival endpoints were markedly superior for the former category.

When attempting to determine whether radiation therapy imparts any survival benefit among resected pancreatic cancer patients using the RTOG 9704 and CONKO-001 trials, it is crucial to note the differences among the patient population enrolled in each trial. Enrollment of definitive R0 resected patients within the RTOG 9704 trial was approximately 50% lower than the CONKO-001 trial. The latter trial also used biomarkers (specifically CEA and CA 19-9) as exclusion criteria, thereby preselecting a patient population clinically destined to have better survival. Low levels of postresection serum CA 19-9 predicts for increased sensitivity to chemoradiotherapy and improved survival [34]. Indeed when CA 19-9 level is less than 90, median overall survival and 3-year survival improved to 23 months and 32%, respectively, among RTOG 9704 patients [34].

IMPRESS Trial

In 2013, a multi-institutional trial was completed examining adjuvant treatment with the winning regimen from RTOG 9704 (gemcitabine plus 5-FU chemoradiation) plus Algenpantucel-L immunotherapy [35], an allogeneic vaccine consisting of two irradiated prostate cancer cell lines reengineered to express the murine α-1,3-galactosyltransferase gene. With a median follow-up of 21 months, 70 patients treated with this combination showed an improvement in 1-year survival to 86% in comparison to 69% in the gemcitabine arm of RTOG 9704 [18]. These results prompted the initiation of the phase 3 IMPRESS (Immunotherapy for Pancreatic Resectable Cancer Study) trial. In this study, 722 patients were randomized 1:1 to gemcitabine with or without 5-FU-based chemoradiation or the same plus Algenpantucel-L. However, the phase 3 trial did not demonstrate a statistically significant difference in overall survival with immunotherapy, which was 33% at 4 years in both experimental and control arms [36].

Nonrandomized Trials

Several nonrandomized trials have been conducted at various institutions across the world in an attempt to improve outcomes among surgically resected pancreatic cancer patients. Recognizing the limitations of any interpretation that can be made from these trials, among which include limited power, patient selection bias, varied inclusion criteria, and diverse treatment protocols, it is worth noting the results of some larger and interesting studies. A retrospective analysis of 1092 patients treated at the Johns-Hopkins Hospital and Mayo Clinic between 1993 and 2005 was performed to determine the benefits of adjuvant radiation among their pancreatic adenocarcinoma patients [27]. Just about 50% of these patients underwent 5-FU-based chemoradiotherapy after surgery, while the rest had no adjuvant therapy. Median overall survival and the 2-year

and 5-year survival among the chemoradiotherapy treated patients was 21.1 months, 44.7% and 22.3% when compared to 15.5 months, 34.6% and 16.1%, respectively, among the nonradiation treated patients. Age, resection margin, T-stage, and nodal status were all crucial factors in improving overall outcome. What is not clear is whether the any proportion of patients within the nonradiation treated cohort received any systemic chemotherapy alone. Recognizing that variation within treatment protocols as well as selection bias of healthier patients receiving more aggressive therapy likely exists in this analysis, it is still interesting to note a marked and sustained improvement in survival among patients who received some duration of radiation in the adjuvant setting.

The ACOSOG Z05031 multicenter phase II trial attempted to determine if an aggressive chemotherapeutic regimen with radiation would not only be feasible but also improve outcome [28]. Eighty-nine patients were enrolled in this trial in which they were treated with continuous infusion 5-FU (200 mg/m^2) with concurrent radiation (50.4 Gy) along with weekly cisplatin (30 mg/m^2) and interferon- radiation*6*] units three times a week. This was then followed by two 6-week cycle of continuous infusion 5-FU (200 mg/m^2). Though the trial did not complete its projected enrollment and had to be terminated early from marked grade 3 toxicity, median overall survival, and the 2-year survival among those that enrolled was 25.4 months and 59%, respectively. Although resection margin status had an impact on survival, it was not statistically significant (R0 vs. R1, median OS 31.9 vs. 18.9 months, $p = 0.103$). Nonetheless, this treatment protocol was deemed too toxic to pursue for phase III evaluation.

Investigators at the Stanford University Medical Center reported on the use of adjuvant chemoradiotherapy using only continuous infusion 5-FU (200–250 mg/m^2) with 54 Gy EBRT [29]. Fifty-two patients were treated and completed this protocol resulting in the median overall survival and the 2-year and 3-year survivals of 32 months, 62% and 39%, respectively. Since no significant toxicities were seen for this protocol, further studies with dose intensification are being proposed. An Italian study conducted a feasibility study using cisplatin (40 mg/m^2), epirubicin (40 mg/m^2), gemcitabine (600 mg/m^2), and continuous infusion 5-FU (200 mg/m^2) followed by radiation therapy [30]. Results confirm tolerability of the regimen, with median overall survival and 2-year and 5-year survivals to be 27 months and 53% and 22%, respectively. The results of this trial are very similar to the ACOSOG Z05031 trial substantiating the use of a 5-FU- and cisplatin-based regimen concurrently with radiation therapy.

A Surveillance, Epidemiology, and End Results (SEER) analysis confirmed the benefit of adjuvant radiation treatment among resected pancreatic cancer patients [37]. More than 3,300 patients were identified from the SEER registry who had undergone surgical resection for nonmetastatic pancreatic cancer between 1998 and 2006. Among these, 48% underwent adjuvant radiation therapy and were found to have a significant improvement in median overall survival when compared to those who did not have radiation therapy (19 vs. 14 months, $p < 0.001$). Use of chemotherapy was not specified for either cohort. Nevertheless, use of adjuvant radiation therapy was determined to be an independent predictor of survival among resected pancreatic adenocarcinoma patients.

Finally, in a retrospective National Cancer Data Base study examining 6165 patients treated with adjuvant chemotherapy ($n = 2334$, 38%) versus chemoradiotherapy ($n = 383$, 62%), the addition of radiation improved median survival from 20 to 22.3 months ($p < 0.001$) [38]. This survival benefit remained significant even when subset analyses were performed for R0, R1, pT3, pN0, or pN1 patient groups.

Conclusion

Based on the results of these trials (Table 1), it is clear that resected pancreatic adenocarcinoma patients have improved survival with adjuvant therapy. Notwithstanding the results of the ESPAC-1 trial, all other studies undoubtedly revealed a marked improvement in median survival and 3- or 5-year survival among their treatment arm when compared to the control arm (usually no adjuvant therapy). Furthermore, apart from the EORTC analysis, all studies, including the RTOG 9704 (when accounting for a similar selection criterion as the CONKO-001), revealed statistically significant benefit in survival when compared to no adjuvant therapy. When accounting for a similar criterion of low postresection serum CA 19-9 level, there was more than 2 month improvement in median survival with the use of radiation in a gemcitabine-based adjuvant protocol. Moreover, data from institutional trials, as well as retrospective analysis and SEER analysis all point to an improvement in both disease free survival and overall survival with the use of radiation in the adjuvant setting. The more recent CONKO-001 and the RTOG 9704 studies corroborate the superiority of a gemcitabine-based chemotherapy over a 5-FU-based regimen. Smaller trials even confirm the feasibility of using gemcitabine concurrently with radiation as well, instead of the continuous infusion of 5-FU used in all of the randomized trials [39–41]. At present, the role of adjuvant therapy in pancreatic cancer continues to evolve. However, based on the available information to date, a gemcitabine-based chemoradiotherapy regimen has been established as the "backbone" therapy upon which future trials will likely be conducted.

Patient selection remains a key factor in both performing adequate clinical trials as well as effectively formulating treatment plans that are appropriate for each pancreatic cancer patient. Further critical analysis of all randomized trials discussed above note that inadequate patient selection is likely the common deficiency of each of them. Indeed, it is argued that every one of these trials had a heterogeneous mix of patients, among which included those who truly had a completely resected disease, some with persistent local disease, and possibly some with micrometastatic disease [42]. Since the overall survival is markedly varied among each of these patient populations, evaluation of the true benefit of the treatment arm for each trial must really be questioned. Indeed, no trial reveals results that extend a profound survival improvement when the treatment arms are compared to each other. Failure to distinguish among these patient populations is likely due to the lack of adequate pre- and postoperative imaging studies, inadequate quality control of surgical techniques, and the lack of quality control in pathological evaluation specifically related to margin status. There is sufficient evidence that incomplete surgical resections lead to median survival rates

comparable to inoperable locally advanced pancreatic cancer [26, 43, 44]. Furthermore, recent data suggests positive surgical margins occur often than previously reported [44–46]. This discrepancy among positive surgical margins exists within the adjuvant trials as well. Even though the ESPAC-1 authors reportedly enrolled a high number of R0 resected patients within their trial, the final results revealed greater than 62% of patients had local recurrence with 35% demonstrating local failure only [25]. This high rate of local recurrence implies that a greater number of true R1 and R2 resections were likely present within the trial. Similarly, the CONKO-001 trial also had high local failure rates (34% among gemcitabine treated patients and 41% among the observation cohort) [20]. This trial also failed to define local failure and had inadequate postoperative follow-up evaluations. The recommendation of a CT scan within 6 months of enrollment likely resulted in late detection of any persistent disease postoperatively. Finally, the RTOG 97-04 trial had a high 33% positive surgical margin rate; however, a pretreatment CT scan and radiation quality control perhaps contributed to the lower local recurrence rates (28% among the 5-FU arm vs. 23% among the gemcitabine arm) in this trial [18]. Lack of radiation quality control among the GITSG, EORTC, and ESPAC-1 trials probably further added to the discrepancies in delivering equivalent adjuvant chemoradiation among each of these trials. Therefore, strict patient selection criteria need to be established in order to determine the true benefits of adjuvant chemoradiation among the true R0 resected pancreatic cancer patients.

Despite evidence from these and many other smaller studies revealing substantial benefit of chemoradiotherapy in the adjuvant setting, long-term prognosis for pancreatic cancer patients continues to remain grim. It is becoming clearer that appropriate patient selection for both surgical resection as well as adjuvant chemoradiotherapy will identify a subpopulation of patients who will benefit the most from such aggressive measures. Further studies need to occur in attempting to delineate various patient populations that separate those with poorer prognosis from others. Future adjuvant trials should employ modern imaging techniques in the preoperative setting to identify the truly resectable versus the locally advanced pancreatic cancer cases, by using specific anatomic determinants [47, 48]. In addition, future trials should also employ strict surgical and pathological quality control along with postoperative imaging in order to further select out specific patients and then interpret the true benefits of adjuvant therapy. Lastly, development of newer chemotherapeutics and biological agents, a better understanding of the basic molecular profile of pancreatic cancer, along with improved radiation techniques (e.g., Intensity Modulated Radiation Therapy) should increase our armamentarium in fighting this deadly disease.

Key Practice Points

- Adjuvant therapy after pancreaticoduodenectomy increases survival
- Using a gemcitabine-based “backbone” chemotherapy is superior to using a 5-flurouracil-based chemotherapy in the adjuvant setting
- Negative surgical resection margins (R0 Resection) lead to improved survival

- Adjuvant combination chemoradiation may provide superior results to chemotherapy alone
- Optimal dose of radiation is considered to be in the range of 45–54 Gy

Key Research Points

- Establish precise pathologic criteria for enrollment into adjuvant therapy clinical trials
- Require accurate pathologic margin status with robust quality control for trial enrollment
- Establish strict quality control measures for delivery of radiation treatments
- Equal randomization of chemotherapy versus chemoradiation to confirm superiority
- Rigorous central review of patient enrollment, optimal delivery of treatment, and eventual reporting of trials data

Future Research Directions

- Utilizing a multidisciplinary approach to the treatment of pancreatic cancer
- Determining the optimal candidate for pancreaticoduodenectomy by establishing strict surgical resection criteria using improved diagnostic radiographs
- Employing innovative radiation techniques, such as IMRT, to minimize toxicity and intensify either radiation or chemotherapy treatments or both
- Utilizing novel chemotherapeutics and biologics based on specific molecular targets
- Improving surgical outcomes utilizing multimodality therapy in the neoadjuvant setting

Published Guidelines

- NCCN Clinical Practice Guidelines in Oncology, *Pancreatic Adenocarcinoma* National Comprehensive Cancer Network, v1.2017 [49].
- ESMO Clinical Practice Guidelines for the diagnosis, treatment, and follow-up, *Pancreatic Cancer,* 2015 [50]
- Japanese Pancreas Society, *Clinical Practice Guidelines for Pancreatic Cancer,* 2016 [51].

Cross-References

► Adjuvant Chemotherapy in Pancreatic Cancer
► Emerging Therapeutic Targets in Pancreatic Adenocarcinoma
► Role of Radiotherapy in Locally Advanced Pancreatic Cancer

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Arterial Resection in Pancreatic Cancer

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Abstract

Pancreatic cancer surgery is the only potentially curative approach for this disease and remains a formidable challenge. Better perioperative management, increased experience and advanced surgical techniques, and centralization of care have significantly reduced morbidity and mortality rates of major pancreatic resection. Together with more active and effective chemotherapeutic and radiotherapeutic regimen, this has led to an increase use of resectional procedures in borderline resectable and locally advanced unresectable tumors. Especially for the latter, arterial resection is often necessary to achieve clear margins. However, this approach is currently under debate with higher rates of complications reported. In this chapter, an overview is provided of potential indications and techniques as well as short- and long-term outcomes associated with these procedures.

Keywords

Pancreatic cancer · Arterial resection · Appleby procedure · Locally advanced · Neoadjuvant therapy

Introduction

Surgical resection is the only potential curative treatment of pancreatic cancer. The pancreas has an abundant and complex vascular supply (Fig. 1). Unfortunately due to this, pancreatic cancers often grow close to or invade the superior mesenteric vein/portal vein or superior mesenteric artery/celiac trunk/hepatic artery [1]. In most cases, this either defines locally advanced unresectable tumors or requires vascular resection to achieve macroscopic tumor clearance, i.e., R0/R1 resections. Arterial resection for pancreatic cancer, however, has been labeled as potentially harmful, by the 2014 consensus statement from the International Study Group of Pancreas Surgery (ISGPS) [2]. Their review of the evidence cited the increased mortality and morbidity of the surgery, without evidence of increased survival in comparison to resection alone. However, a head-to-head comparison of tumor resection with arterial resection versus *no* resection has not been carried out. The consensus view

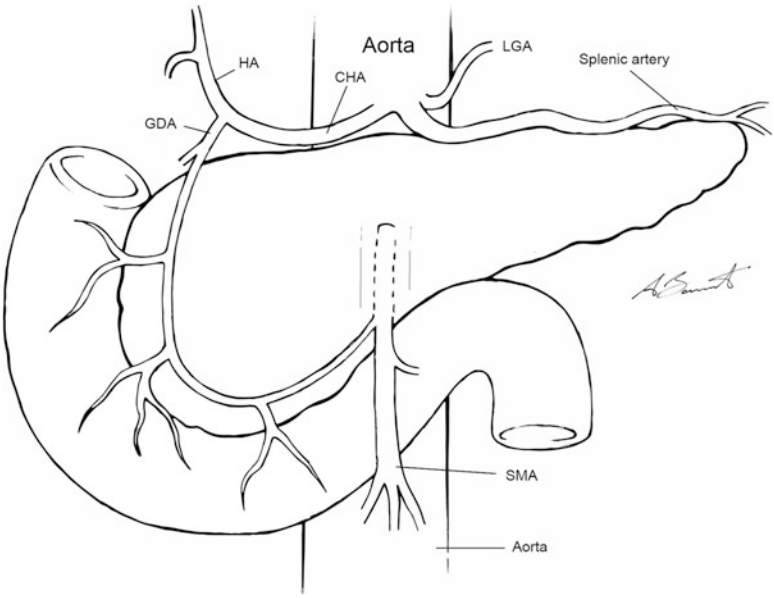

Fig. 1 Arterial supply of the pancreas. *HA* hepatic artery, *CHA* common hepatic artery, *LGA* left gastric artery, *GDA* gastroduodenal artery, *SMA* superior mesenteric artery

is that definitive arterial involvement should be considered locally advanced unresectable and thus managed with palliative intent in most cases [2, 3]. This however is based on the available evidence, which includes just a few hundred reported cases in the literature, mostly from small retrospective case series conducted over a long time period [4], and recent data has challenged this view with reported resectability rates of 60% in initially locally advanced unresectable cases that underwent induction FOLFIRINOX therapy [5]. As such, a degree of caution has to be applied to the conclusions, allowing for variation in approach taking into account more recent data and novel multimodal and technical approaches.

Despite the ISGPS consensus, arterial resections for pancreatic cancer have been increasingly published [3, 6–8]. However, these are not reporting the standard of care in most centers worldwide, but an available approach in highly selective individuals. While routine arterial resection is not advocated, it may be appropriate for a selected group of patients. This group may become more prevalent as more effective methods of tumor downstaging become available and experience with the technical challenges advances. The best predictor of survival following a diagnosis of pancreatic cancer is a successful (R0) resection [9], although R1 resections are also effective in providing long-term benefit together with adjuvant therapy [10, 11]. While rare, there is some reported long-term survival following arterial

resection [12]. Here an overview is presented on arterial resection following pancreatic cancer surgery, including indications, technical aspects, and outcome.

Preoperative Assessment and Patient Selection

The need for arterial resection should ideally be identified preoperatively [3], and all such cases should be managed in high-volume centers, within specialist multidisciplinary teams. This management should include high-quality CT scanning with mandatory pancreatic protocol to assess resectability. The National Comprehensive Cancer Network (NCCN) guidelines of CT classification of resectability have largely gained acceptance (Table 1) and were supported by the ISGPS in 2014 [2]. All cases where there is a possible need for arterial reconstruction should therefore be considered either locally advanced, unresectable (most cases), or borderline resectable on preoperative imaging [2, 3].

The potential need for arterial resection should prompt consideration of a different management approach. Arterial infiltration by pancreatic cancer can be seen as a marker of a biologically aggressive tumor [2, 4, 13], although this has not been convincingly proven on a molecular level. Presumed vascular involvement is not confirmed in a relevant proportion intraoperatively or on histology, especially after neoadjuvant therapy. Further, tumor cells tend to grow along nerve plexuses around the superior mesenteric artery/celiac trunk without true infiltration [14]. Where

Table 1 NCCN/ISGPS guidelines defining resectability status [2]

Localized and resectable	Borderline resectable	Locally advanced, unresectable[a]
No distant metastasis	No distant metastasis	No distant metastasis
No radiographic evidence of SMV or PV distortion	Venous involvement of the SMV or PV with distortion or narrowing of the vein or occlusion of the vein with suitable vessel proximal and distal, allowing for safe resection and replacement	Unreconstructible SMV/portal occlusion
Clear fat planes around CA, HA, and SMA	GA encasement up to the hepatic artery with either short segment encasement or direct abutment of the HA without extension to the CA	Any celiac abutment
	Tumor abutment of the SMA not to exceed 180° of the circumference of the vessel wall	Greater than 180° SMA encasement
		Aortic/IVC invasion or encasement

CA celiac axis, *GA* gastroduodenal artery, *HA* hepatic artery, *IVC* inferior vena cava, *NCCN* National Comprehensive Cancer Network, *PV* portal vein, *SMA* superior mesenteric artery, *SMV* superior mesenteric vein

[a]Criteria are given only for cancers of the head

vessels are truly involved in pancreatic cancer, the cancer tends to infiltrate along the intimal surface of the vessels [15].

Studies investigating the use of arterial resection have suggested reservation of the technique for those less likely to develop (or harbor) systemic disease. Consequently most clinicians would advocate neoadjuvant therapy in these patients [16–19], even though there is insufficient evidence to recommend neoadjuvant therapy in resectable or borderline resectable patients [2]. One accepted rationale of neoadjuvant therapy is to select those cases with systemically progressing tumors who would not benefit from major resectional surgery.

Those advocating neoadjuvant therapy in locally advanced unresectable or borderline resectable pancreatic cancer with suspected arterial involvement have utilized a variety of chemotherapeutic agents and radiotherapy protocols [16–19], with different durations and doses, consequently making it very difficult to offer evidence based recommendations. Ideally where neoadjuvant therapy is undertaken in such patients, it should be in the context of clinical trials, so that suitable regimens can be identified. Clinical trials in progress may provide further clarification on this issue (e.g., ESPAC-5F or NEOPAN).

Where neoadjuvant therapy has been undertaken, it is considered important to operatively explore all patients in whom disease remains localized without evidence of metastatic spread [2]. This is following several reports suggesting that post-therapy changes after neoadjuvant treatment are currently not distinguishable from neoplastic disease on imaging and that R0 resection may be possible on surgical exploration even in cases that remain formally unresectable on restaging [20–22].

When formulating a preoperative plan in a patient where arterial resection is planned, the need for concurrent portal vein venous resection is an important component of the plan [23]. This is especially true for lesions where hepatic artery or coeliac axis resection is being planned. Given the dual blood supply of the liver, reconstruction of the hepatic artery may not always be necessary, especially in cases where gastroduodenal arterial flow is preserved or where aberrant arterial anatomy preserves a degree of hepatic arterial flow (e.g., replaced right hepatic artery) [16]. However, where concurrent portal vein resection is undertaken, there should be a lower threshold for performing arterial reconstruction [23]. This is because during the vein resection the liver will suffer an ischemic insult, and arterial compromise could exacerbate the insult. For this reason where resection (with reconstruction) of both arterial and portal supply to the liver is planned, it should be performed in a sequential manner, to minimize the ischemic insult [24, 25].

Arterial Resections

There are two broad situations where arterial resection may be considered in pancreatic cancer surgery. Coeliac axis resection may be undertaken in left-sided pancreatic resections and in right-sided pancreatic resections either common hepatic artery resection or superior mesenteric artery resection.

Coeliac Axis Resection in Left-Sided Pancreatic Resections

Unfortunately most patients with distal pancreatic cancer present at a later stage of disease, meaning that tumor involvement of the coeliac axis is not uncommon [19]. However, it is possible to resect the distal pancreas with the entire coeliac axis (DP-CAR) without reconstruction, due to the development of collateral supply via the gastroduodenal artery [19, 26]. The procedure was first described in 1953, by Lyon Appleby, as part of a resection undertaken for an advanced gastric cancer necessitating en bloc distal pancreatectomy [27]. A modification of this procedure shown in Fig. 2 has subsequently been employed to facilitate achieving an R0 resection in highly selective cases [8, 18, 28–30].

The DP-CAR is the most commonly performed arterial resection in pancreatic cancer [4]. Despite this, just 240 cases over a time period of 1975–2014 were identified in a recent systematic review [26]. Many of these studies were conducted over wide time periods, in highly varied clinical settings, meaning that their relevance to modern practice is limited. The most recent large series reported only 20 DP-CAR cases within an overall series of 822 patients undergoing a distal pancreatectomy [31].

Methods reported to decrease the ischemic complications of coeliac artery resection include preoperative embolization of the common hepatic artery (PHAE) and reports of laparoscopic ligation of the coeliac axis [32–34]. However, a recent systematic review of DP-CAR identified that only 55 of 155 cases (where it was reported) underwent PHAE, with ischemic complications occurring in 21 of 233 cases, with no obvious identified benefit in the PHAE patients [26]. There are limited reports of DP-CAR with hepatic arterial reconstruction, and it has been advocated that reconstruction should be guided by the drop of flow within the hepatic artery following coeliac axis clamping as measured by common hepatic artery pressure or if there is a loss of biphasic arterial flow [16, 34]. An intraoperative measurement of the intrahepatic blood flow using a duplex ultrasonography will certainly have a role in this. A suggested level at which arterial reconstruction should be considered was a reduction in pressure by 25% following coeliac axis clamping [34]. Currently however there is insufficient data to support adoption of this technique, especially given the potential risk for increased vessel trauma. Approaches to arterial reconstruction are discussed below, as they are most frequently necessitated in right-sided pancreatic resections [4].

The most recent series report 30-day mortality for DP-CAR at 10% compared to 1% in standard distal pancreatectomy [31]. This higher mortality is at odds with systematic reviews suggesting a perioperative mortality of 3.0–3.5% [8, 26]. Of note, median hospital stay is reported at 32 days, which is much higher than typical pancreatic cancer resections [26], especially for left-sided tumors. Despite the limited evidence base, it is reasonable to accept that a DP-CAR is associated with a significant increase to the risk of perioperative mortality, though the exact level of this is difficult to quantify, and will be highly dependent on individual cases and institutions.

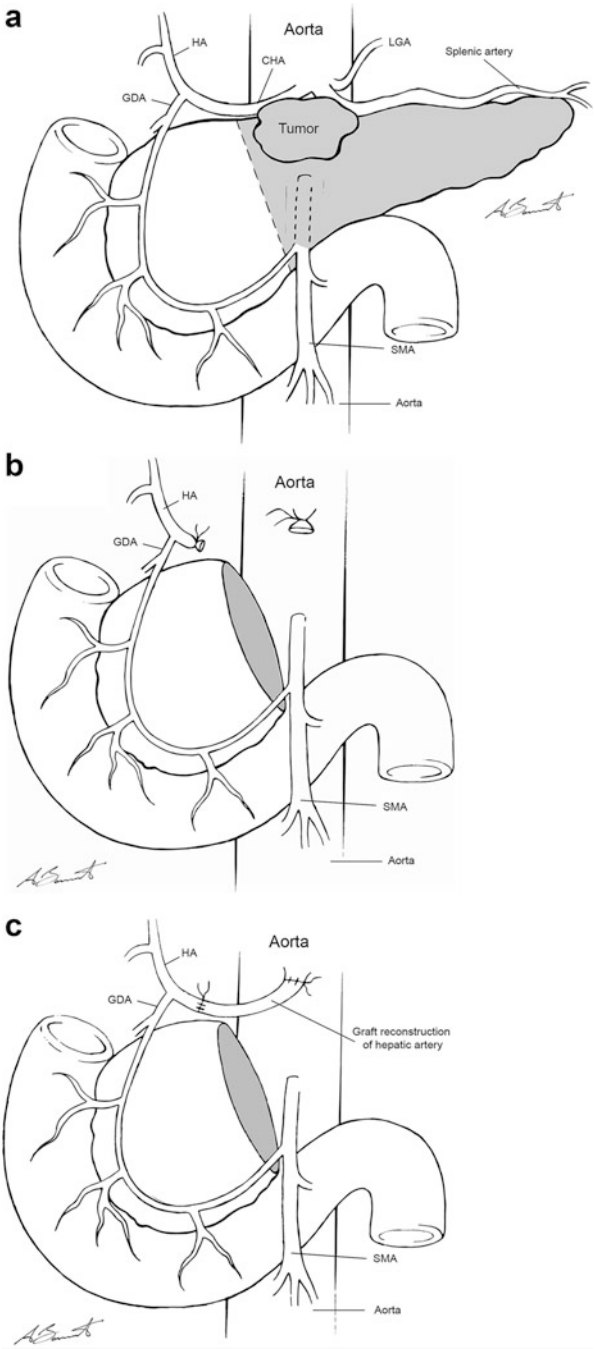

Fig. 2 Schematic of Appleby resection including coeliac axis (**a**). Appleby resection without reconstruction (**b**). Appleby resection with graft reconstruction of hepatic artery (**c**). *HA* hepatic artery, *CHA* common hepatic artery, *LGA* left gastric artery, *GDA* gastroduodenal artery, *SMA* superior mesenteric artery

A large multicenter study compared DP versus DP-CAR. In this study, overall morbidity was comparable (36% vs. 35%) [31]. Major complication following DP-CAR can be expected at a rate of approximately 30% of patients [26]. Of particular concern following DP-CAR are ischemic complications that are not typical for standard DP. These include complications ranging from ischemic gastropathy to gastric, hepatic, or gallbladder necrosis [26]. The overall rate of ischemic complications is seen in around 8% of patients [26].

Achieving an R0 resection is seen as the primary aim of the extended arterial resection in pancreatic cancer. This was achieved in 152 of 204 of cases, which is lower than could be expected in typical distal pancreatectomies [35]. However, given this is locally advanced, irresectable disease by definition an R0 rate of approaching 75% can be seen as a significant technical achievement [26].

The primary advantage of a DP-CAR is achieving an R0 resection; however, one potential advantage of the approach has been the potential improvement in the typical epigastric pain seen in many patients [28, 36]. The intractable pain in pancreatic cancer is likely due to tumor involvement of the coeliac plexus and coeliac ganglions. These are resected in a modified Appleby procedure. It is thought that the improved pain is mediated through this mechanism [36]. Unfortunately, the majority of studies do not report on quality of life measures, so meaningful analysis of the affect this may have on overall quality of life is not possible.

Arterial Resection in Right-Sided Pancreatic Cancer Resections

The majority of patients undergoing curative intent surgery for pancreatic cancer have right-sided pancreatic lesions [9]. However, when examining patients undergoing arterial resection for pancreatic cancer, the majority tend to have left-sided resections [4]. The proximity of major vascular structures to the right-sided pancreatic lesions means that arterial involvement is not uncommon and has been defined as a reason for unresectability [2, 37, 38]. Indeed, more tumors are borderline resectable or locally advanced unresectable and then resectable at the time of presentation.

Arterial resections are much less frequently performed in patients undergoing right-sided pancreatic resection [4, 6]. In the published literature, only a third of patients undergoing arterial resection have a right-sided resection [4, 6]. This is due to the increased complexity of arterial resection in association with pancreatic head resection [24]. Arterial resection in right-sided pancreatic lesions typically necessitates reconstruction to restore arterial flow in either the superior mesenteric artery or common hepatic artery, so as to prevent catastrophic ischemic complications [39, 40]. One such resection and reconstruction technique is demonstrated in Fig. 3. Occasionally due to aberrant hepatic arterial anatomy,

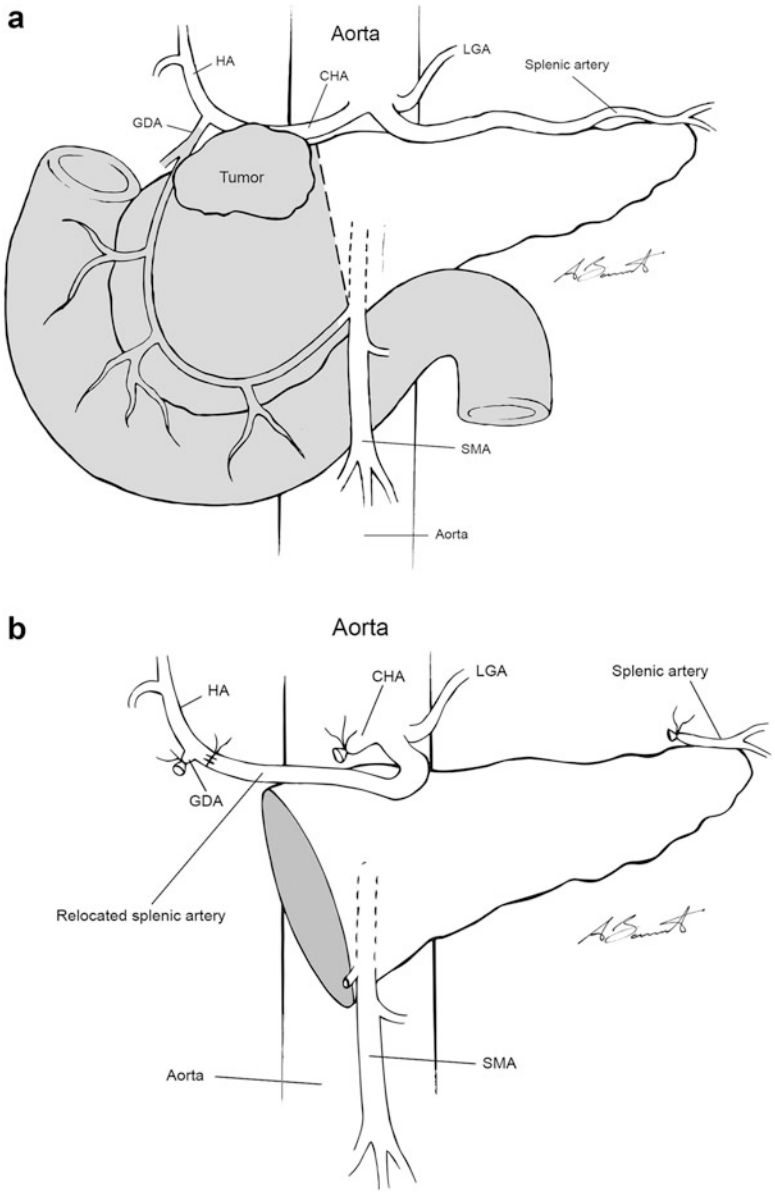

Fig. 3 Schematic of pancreas head resection including common hepatic artery (**a**). One possible reconstructive technique where splenic artery is mobilized to reconstruct common hepatic artery (**b**). *HA* hepatic artery, *CHA* common hepatic artery, *LGA* left gastric artery, *GDA* gastroduodenal artery, *SMA* superior mesenteric artery

resection can be performed without reconstruction such as in cases of a replaced right hepatic artery [25].

Very few studies have concentrated on arterial resection and reconstruction in right-sided pancreatic cancer, and consequently the evidence base looking at post-operative morbidity and mortality is limited [2, 24].

Arterial Reconstruction Techniques

A variety of approaches to arterial reconstruction have been applied [4]. The three main approaches include primary reconstruction with end-to-end anastomosis, vein interposition grafts, and arterial interposition grafts [4]. Arterial bypass techniques have also been performed, but typically this is for inadvertent arterial injury during pancreatic resection [12].

Primary End-to-End Reconstruction

The most commonly performed arterial reconstruction is primary resection and end-to-end anastomosis [4]. Performing an end-to-end reconstruction requires suitably mobile arterial lengths that are comparable in size. This is so that an anastomosis can be constructed without tension. Consequently end-to-end primary repairs are typically suitable for small segment arterial involvement and resection.

Vein Interposition Graft

When primary repair is not feasible, such as in extensive vascular resections, venous interposition grafts can be performed. Typically the saphenous vessels are harvested and arterial flow restored [25, 41]. These can either be bridging the gap of resected vessels or by creating an alternative flow from neighboring vessels such as the left gastric artery or from the SMA to the CHA [25, 34]. They can also be reconstructed directly from the aorta.

Artery Interposition Graft

Where primary end-to-end arterial anastomosis is not possible, arterial interposition grafts can be carried out. These are typically autografts, with the use of splenic artery or internal iliac artery [24]. The use of splenic artery as an interpositional graft has been reported in case of pancreatic body carcinoma with the involvement of coeliac axis, common hepatic artery, and the gastroduodenal artery, where a total pancreatectomy with coeliac axis resection is required [42]. When autografts are not used, cryopreserved blood type matched vessels can be another option [6].

Perioperative Management

When performing arterial resection in pancreatic cancer, there are a number of specific complexities that need to be considered. Currently the evidence base upon which clinical practice can be guided is limited, and much of the evidence to guide practice in such cases is taken from other areas of clinical practice.

Even brief periods of hypotension could lead to severe consequences. These could be induced if there is further ischemic compromise of intra-abdominal organs in cases without reconstruction or through inducing thrombosis in cases with arterial reconstruction. Unfortunately intra-abdominal ischemia may be difficult to distinguish from postoperative pain, and the opportunity to intervene may be missed before the diagnosis is made [43, 44]. To aid early diagnosis, it is essential to maintain a low index of suspicion and have access to high-quality CT angiography and ultrasound duplex imaging at all times [43]. Centers should not undertake cases without 24-h access to such imaging modalities and access to full compliment of interventional radiological techniques.

In patients with an arterial reconstruction, an early thrombosis represents a major perioperative risk, with likely high mortality and morbidity [43]. Consequently, therapeutic anticoagulation may be considered, though this may increase the risk of postoperative bleeding [45]. It may be that anticoagulation can be reserved for those deemed to be at high risk of thrombosis. In the transplant setting, the use of blood transfusion and technical challenges in the resection have been identified as risk factors for concerns [43]. A focus of future research should be to identify the optimal patients and regimen for postoperative anticoagulation. Currently, management must be based on clinical judgment in individual cases, with therapeutic anticoagulation reserved for those deemed to be at higher risk of thrombotic complications.

A final consideration is the presence of other nonvascular anastomoses. In right-sided pancreatic resections, there are typically a number of anastomoses, in comparison to left-sided resections where they are not typical [46]. Anastomotic leak is one of the most prevalent significant complications following pancreatic resection and a major source of morbidity and mortality [47]. Arterial resection could be seen as putting patients at increased risk of anastomotic breakdown given the increased blood loss, longer operative time, and the ischemic insult during the resection [4]. Anastomotic breakdown is also a risk factor for increased delayed postoperative bleeding [43]. These factors may underpin the higher reoperation rates in patients undergoing arterial resection [4]. When performing major vascular resection, a total pancreatectomy may be preferable, to avoid the need for a pancreatic anastomosis and hence increase its associated morbidities

Prognosis

Very few studies report on 5-year survival for patients undergoing arterial resection in surgery [4, 26, 28, 40, 48, 49]. The few studies reporting it offer 5-year survival of between 0% and 15% [2]. Median survival is reported in more studies and typically

is reported at between 12 and 22 months, with weighted median survival in DP-CAR reported at 14.4 months [4, 26]. This survival must obviously be viewed in context.

It would be unfair to compare such survival with the overall survival of patients with minimal histological or clinical features predictive of poor outcome. Indeed work has suggested that patients with very favorable clinical and pathological features can have 5-year survival approaching 60% [50]. The need for an arterial resection should be seen in the context of advanced disease, and comparisons if any should be drawn from other locally advanced and unresectable cases. In patients with inoperable pancreatic cancer at laparotomy with or without bypass procedures, who go on to have palliative chemotherapy, median survival is 14.4–16.3 months [51], which seems to be superior to patients who undergo palliative (i.e., R2) resection, underlining the key requirement of obtaining an R0 resection when considering arterial resection [52]. Further, a recent meta-analysis of locally advanced pancreatic cancer patients treated with FOLFIRINOX and of whom only around 25% were resected, median survival was 24.2 months [53]. However, this is comparing data of most effective chemotherapy with advanced surgery without therapy or with less effective therapy. Obviously, the best available therapy plus advanced and safe surgery should be put into the equation. When the median survival of patients undergoing arterial resection is viewed in this light, it does not appear as bleak.

Importantly resection remains the only curative treatment for pancreatic cancer, and when offered to patients, that small chance of survival may to an individual be deemed worth the surgical risk.

Conclusion

Arterial resection should only be considered in a highly selected group of physically fit patients and in patients where there is a high chance of obtaining an R0 resection [52]. Centers performing such surgery should carry out a high volume of major pancreatic resections and have a comprehensive multidisciplinary approach and support for their service. In particular, 24-h access to a full compliment of radiological imaging and intervention should be seen as essential.

Patients should probably only undergo resection if disease is stable or responding to neoadjuvant therapy, without evidence of distant metastases. All patients should be considered for current clinical trials to further the evidence base for such resections. While these are a highly selective group of patients at present, as the chemotherapeutic armamentarium advances, it is likely that the frequency for combined pancreatic and arterial resections will increase.

Cross-References

► Borderline Resectable Pancreatic Cancer
► Clinical Decision-Making in Pancreatic Cancer

- ▶ Neoadjuvant Chemotherapy in Pancreatic Cancer
- ▶ Surgical Resection for Pancreatic Cancer Using the International Study Group of Pancreatic Surgery (ISGPS) Classifications
- ▶ Venous Resection in Pancreatic Cancer Surgery

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Treatment of Recurrent Pancreatic Cancer After Surgery

Oliver Strobel, Willem Niesen, and Markus W. Büchler

Contents

Abstract

The majority of patients with pancreatic cancer eventually develop and die from recurrence even after successful surgical resection and adjuvant therapy. Pancreatic cancer recurrence and its treatment are, therefore, very relevant clinical concerns. For several reasons there is a striking lack of knowledge and evidence with respect to the incidence and pattern, the detection, and the management of pancreatic cancer recurrence. This chapter summarizes available data on the incidence, timing, and pattern of recurrence, discusses the need for and the potential of structured surveillance programs, and provides an overview of treatment options for pancreatic cancer recurrence. While most patients will eventually die from systemic recurrences, a relevant subgroup of 20–30% of

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patients at first present with isolated local recurrence. For systemic recurrences chemotherapy is the only treatment option. However, data from observational cohort studies suggest that treatment strategies that include local approaches may be associated with prolonged survival patients with isolated local recurrences. In order to improve the treatment of both local and systemic recurrence of pancreatic cancer and to enable clinical trials, it will be important to establish surveillance programs after resection and to address treatment options for recurrence in future guidelines.

Keywords

Pancreatic cancer · Resection · Surveillance · Recurrence · Isolated local recurrence · Systemic recurrence · Re-resection · Outcome · Survival

Abbreviations

CA 19-9	Carbohydrate antigen 19-9
CT	Computed tomography
PDAC	Pancreatic ductal adenocarcinoma
PET	Positron emission tomography
RCT	Randomized controlled trial

Introduction

Management of pancreatic ductal adenocarcinoma (PDAC) recurrence is a very relevant topic because even after successful resection and administration of adjuvant therapy, PDAC recurs in the majority of cases. Most patients eventually succumb to local, metastatic, or combined tumor recurrences resulting in a median survival of only 20–25 months and 5-year survival rates around 20% [1, 2]. Three main reasons contribute to the high recurrence rate and poor prognosis of PDAC:

(i) An obvious reason for local recurrences is insufficient resection margin clearance reflected by the high rate of R1 resections identified by stringent margin assessment [3–6]. The high rates of R1 resection are not caused by inappropriate surgical technique but explained by the tumor biology of PDAC with extrapancreatic and extratumoral perineural spread toward the arteries identified in 60–70% of cases [7].

(ii) Even more importantly most patients die from early metastatic recurrence. Undetectable micrometastatic disease at the time of resection is thought to be the main reason for this systemic failure. While this provides a clear rational for the administration of systemic therapies in the adjuvant or neoadjuvant settings (see chapters "▶ Adjuvant Chemotherapy in Pancreatic Cancer" and "▶ Neoadjuvant Chemotherapy in Pancreatic Cancer"), the follow-up data derived from randomized controlled trials on adjuvant therapy show that the tested therapy regimens can significantly delay but not prevent recurrence (see Table 1) [8–17].

Table 1 Incidence, timing, and pattern of recurrence after resection for pancreatic cancer in selected RCTs on adjuvant therapy

Reference and name of study	Study arms	n	Overall survival (median and survival rates)	Disease-free survival (median and survival rates)	Incidence and pattern of recurrence	Follow-up (median)
Neoptolemos et al. (2004) [8] ESPAC-1	4 × 4 factorial design:					Survivors: 47 months
	CRT (20Gy + FU)	73	13.9 months, 5YSR: 7%	Chemotherapy: 15.3 months	Local only: 35%	
	Chemotherapy: FU	75	21.6 months, 5YSR: 29%		Local and systemic: 27%	
	Chemotherapy + CRT	72	19.9 months, 5YSR: 13%	No chemotherapy: 9.4 months	Systemic only: 34%	
	Observation	69	16.9 months, 5YSR: 11%			
Smeenk et al. 2007[a] [9] EORTC 40891 (long-term results)	CRT (40Gy + FU)	110	21.6 months	18 months	Total: 68%	Overall: 11.7 years
			5YSR: 25%, 10YSR: 17%	5YSR: 21%, 10YSR: 16%	Initially local only: 20%	
	Observation	108	19.2 months	14.4 months	Local and systemic: 29%	Survivors: 9.8 years
			5YSR: 22%, 10YSR: 18%		Initially systemic: 48%	
				5YSR: 20%, 10YSR: 17%	Total: 70%	
					Initially local only: 21%	
					Local and systemic: 30%	
					Initially systemic: 46%	
Oettle et al. (2007) [10] CONKO-001	Gemcitabine	179	22.1 months	13.4 months	Total: 74.3%	53 months
			2YSR: 47.5%, 5YSR: 22.5%	2YSR: 30.5%, 5YSR: 16.5%	Local ± systemic: 34%	
	Observation	175	20.2 months	6.9 months	Systemic only: 56%	
			2YSR: 42%, 5YSR: 11.5%	2YSR: 14.5%, 5YSR: 5.5%	Total: 92.0%	

(*continued*)

Table 1 (continued)

Reference and name of study	Study arms	n	Overall survival (median and survival rates)	Disease-free survival (median and survival rates)	Incidence and pattern of recurrence	Follow-up (median)
Regine et al. (2008) [11] RTOG 97-04	FU – CRT (FU, 50.4 Gy) – FU	230	16.9 months, 3YSR: 22%	NA	Total: 85.7%	Overall: 1.5 years
					Local: 28%, regional: 8%,	
					systemic: 71%	
	Gemcitabine – CRT (FU, 50.4Gy) – Gemcitabine	221	20.5 months, 3YSR: 31%	NA	Total: 83.3%	Survivors: 4.7 years
					Local: 23%, regional: 7%	
					Systemic: 71%	
Ueno et al. (2009) [12] JSAP-02	Gemcitabine	58	22.3 months	11.4 months	Total: 76%	60.4 months
			2YSR: 48.3%, 5YSR: 23.9	2YSR: 27.2%	Local: 23%	
					Systemic: liver 30%, peritoneal 18%, other 27%	
	Observation	60	18.4 months	5.0 months	Total: 88%	
					Local: 32%	
			2YSR: 40.0%, 5YSR: 10.6%	2YSR: 16.7%	Systemic: liver 30%, peritoneal 13%, other 23%	
Neoptolemos et al. (2010) [13] ESPAC-3	FU + folinic acid	551	23.0 months, 2YSR: 48.1%	14.1 months, 2YSR: 30.7%	Total: 63% (local, systemic or both)	Survivors: 34.2 months
	Gemcitabine	537	23.6 months, 2YSR: 49.1%	14.3 months, 2YSR: 29.6%		
Van Laethem et al. (2010)[b] [14] EORTC-40013-22012/FFCD-9203/ GERCOR	Gemcitabine (4 cycles)	45	24.4 months, 2YSR: 50.2%	10.9 months	Local only: 24%	33.3 months
					Local and systemic: 13%	
					Systemic only: 40%	
	Gemcitabine, (2 cycles) + Gem-based CRT	45	24.3 months, 2YSR: 50.6%	11.8 months	Local only: 11%	30.7 months
					Local and systemic: 20%	
					Systemic only: 42%	

Schmidt et al. (2012) [15] CapRI	Chemoradioimmunotherapy (FU, cisplatin, interferon, 50 Gy)	64	32.1 months	15.2 months	Total: 67% (local, systemic or both)	Overall: 42.7 months
	FU + folinic acid	68	28.5 months	11.5 months		
Uesaka et al. (2016) [16] JASPAC 01	Gemcitabine	190	25.5 months	11.3 months	Total: 67%	82.3 months
			3YSR: 38.4%,	3YSR: 22.6%,	Local: 26%	
			5YSR: 24.4%	5YSR: 16.8%	Systemic: liver 29%, peritoneal 16%, other 32%	
	S1	187	46.5 months	22.9 months	Total: 66%	79.3 months
			3YSR: 59.0%, 5YSR: 43.6%	3YSR: 39.2%, 5YSR: 33.3%	Local: 19%	
					Systemic: liver 19%, peritoneal 12%, other 33%	

Updated from Strobel and Büchler [17]

ESPAC European Study Group for Pancreatic Cancer, *EORTC* European Organization for Research and Treatment of Cancer, *CONKO* Charite Onkologie, *RTOG* Radiation Therapy Oncology Group, *YSR* year survival rate, *FU* fluorouracil, *NA* data not available, *JASPAC* Japan Adjuvant Study Group on Pancreatic Cancer

[a]Only T1/2, N0-1a pancreatic orT1–3, N0-1a periampullary cancers included

[b]Only R0-resections included

(iii) The aggressive tumor biology and high chemoresistance of PDAC are thought to be main reasons for the failure of available regimens for adjuvant therapy to achieve a sustained local and systemic control [2].

With significant improvements in the surgical therapy and in accompanying (neoadjuvant or adjuvant) systemic treatment options, the long-lasting controversy on the role of surgery in resectable PDAC has been resolved [1]. High-volume centers have reported actuarial 5-year survival rates after resection of 20% overall and of up to 60% in patient subgroups with a favorable combination of prognostic factors [6, 18–20]. More recently, the JASPAC-1 study has marked a significant advance in adjuvant treatment with S1 resulting in a 5-year survival rate of 44% [16]. Today it is undisputed that surgical resection in combination with systemic treatment remains the only chance of long-term survival or cure in patients with primary PDAC.

In contrast, although PDAC recurrence is a pressing problem affecting the majority of resected patients, its management is poorly studied and highly controversial. A part of the underlying problem is a certain therapeutic nihilism toward PDAC recurrence that is reflected by the fact that most current treatment guidelines do not recommend structured surveillance programs after resection due to a lack of evidence for effective treatment options for recurrence or lack of a survival benefit by regular follow-up exams (see Table 2) [21–26]. Of note, some current guidelines do not even address the problem of PDAC recurrence and its management.

This chapter aims to provide an overview of current treatment options for PDAC recurrence with a special focus on isolated local recurrence. The chapter also addresses several aspects that are relevant in the context of PDAC recurrence, including incidence and pattern of recurrence after resection, and the potential value of structured surveillance after resection.

Incidence and Pattern of Recurrence

The knowledge about the incidence, timing, and pattern of recurrence is vague as surveillance programs are not generally recommended in current clinical guidelines resulting in a lack of follow-up data from large patient cohorts. The best information on clinically detected recurrence is probably available from randomized controlled trials on resection and adjuvant therapy (Table 1 [8–16]**)** with some additional data available from the few observational studies dedicated to the topic of recurrence [27]. A few available autopsy series provide important data on the pathological pattern of recurrence after resection [28–30].

Data from randomized controlled trials (RCTs) provide the best indication of the "clinical" pattern of recurrence detectable by structured follow-up programs with assessment of patient history, physical examination, cross-sectional imaging (usually contrast-enhanced computed tomography (CT)), and serum values of tumor markers,

Table 2 Recommendations on surveillance after resection for pancreatic cancer in selected recent clinical guidelines

Guideline	Recommendation	Level of recommendation	Level of evidence
AWMF Germany 2013 [22]	*9.33*:	A	5
	Structured surveillance programs for PDAC are not recommended as there are no available data that regular staging examinations are associated with a survival benefit	Consistent level 1 studies *According to Oxford Centre for Evidence-Based Medicine*	Expert opinion without explicit appraisal or based on physiology, bench research, or "first principles"
NCCN USA (2016) [21]	*MS-43/PANC-6*:	Category 2B	Lower level
	History and physical examination for symptom assessment, CA 19-9 testing, and follow-up CT scans every 3–6 months, then every 6–12 months	Based upon lower-level evidence, there is NCCN consensus that the intervention is appropriate	
	Are category 2B recommendations, because data are not available to show that earlier treatment of recurrences leads to better patient outcomes		
ASCO USA (2016) [23]	*6.1*:	Moderate	Low
	In the absence of RCT evidence, the panel recommends that patients who have completed treatment of potentially curable pancreatic cancer and have no evidence of disease be monitored for recovery of treatment-related toxicities and recurrence. Visits may be offered at 3- to 6-month intervals; the role of serial cross-sectional imaging, the extent to which surveillance intervals should be prolonged over time, and the duration of recommended surveillance are all undefined	Informal consensus, benefits outweigh harms The available evidence was deemed insufficient to inform a recommendation to guide clinical practice. The recommendation is considered the best current guidance for practice, based on informal consensus of the expert panel	Low confidence that the available evidence reflects the true magnitude and direction of the net effect. Further research may change either the magnitude and/or direction this net effect

(*continued*)

Table 2 (continued)

Guideline	Recommendation	Level of recommendation	Level of evidence
ESMO Europe (2015) [24]	There is no evidence that regular follow-up after initial therapy with curative intent is useful	D	IV
		Moderate evidence against efficacy or for adverse outcome, generally not recommended	Retrospective cohort studies or case-control studies
NCI USA (2016) [25]	Not addressed	NA	NA
IAP and EPC consensus review of guidelines (2015) [26]	Not addressed	NA	NA

AWMF Arbeitsgemeinschaft der Wissenschaftlichen Medizinischen Fachgemeinschaften e.V. (Version 1.0 October 2013), *NCCN* National Comprehensive Cancer Network (Version 2.2016), *ASCO* American Society of Clinical Oncology (2016), *ESMO* European Society for Medical Oncology (2015), *NCI* National Cancer Institute, *IAP* International Association of Pancreatology, *EPC* European Pancreatic Club

especially of carbohydrate antigen 19-9 (CA 19-9). The follow-up results of selected RCTs on resection and adjuvant therapy are summarized in Table 1. These data allow several important conclusions on incidence, timing, and pattern of recurrence after resection for PDAC. Data on disease-free survival from RCTs comparing adjuvant therapy versus observation show that without adjuvant therapy 50% of patients develop clinically detectable cancer recurrence within 5–10 months [8, 10, 12]. Adjuvant chemotherapy with gemcitabine or 5-fluorouracil [5-FU] monotherapy cannot prevent but delay recurrence to 11–15 months. With patient selection based on known prognostic factors, recurrence is observed later, at 14.4 months without and at 18 months with adjuvant therapy [9]. Even in the more recent RCTs, the median disease-free survival remains at 12–15 months [13, 15]. Up to 90% of patients without and about 70% with adjuvant therapy develop PDAC recurrence within a follow-up time of 30–50 months. More recently, the JASPAC-1 study marked an exceptional advance, at least for Asian patients, with a median disease-free survival of 22.9 months and 5-year disease-free survival rate of 33.3% after resection and adjuvant therapy with S1 [16].

While the reporting on the pattern of recurrence in different RCTs is rather heterogeneous and the majority of patients presents with systemic progression, 20–30% of patients are consistently found to primarily present with isolated local recurrence (Table 1). In summary, the data on recurrence from RCTs demonstrate that even with adjuvant therapy, most patients develop recurrence within 1.5 years after resection. The data also suggests that based on structured surveillance programs, it may be possible to identify a subgroup of 20–30% of patients who first develop isolated local recurrence (as detectable by current imaging technology).

A multicenter observational study in 1130 patients undergoing resection between 2000 and 2010 reported a median actuarial overall survival of 25.9 months (median follow-up 18 months) [27]. Based on radiographic evidence, pathologic confirmation, and/or tumor marker elevation, the local recurrence rate in this study was 22%, and metastatic recurrence was detected in 41% of patients, confirming the clinically detectable recurrence patterns observed in RCTs. The identification of positive lymph node status as most relevant risk factor for local recurrence [27] suggests that many patients with "local recurrence" may in fact have progression of pre-existing lymph node metastases and may be good candidates for re-resection.

Only the few available autopsy series can demonstrate the "true" pathological pattern of recurrence and the relevance of the sites of recurrence for death. In an autopsy study in 24 patients who died after resection of pancreatic cancer, 75% of patients had local recurrence, 75% had distant metastases, and the local recurrence was the cause of death in 17% of patients [28]. Another autopsy study in patients with PDAC included 22 patients after resection [29]. At autopsy, two patients (9%) had died of unrelated causes and had no evidence of recurrence, three (14%) had isolated local recurrence, four (18%) had only metastatic recurrence, and 13 (59%) had both local and systemic recurrence. In this study, expression of DPC4 in the tumor was highly correlated with metastatic but not with localized disease [29]. These autopsy studies confirm that after resection and adjuvant therapy for pancreatic cancer, most patients die from systemic disease, but a subgroup of patients develop and die from isolated local recurrence, and molecular properties of the tumor appear to contribute to the pattern of recurrence.

It will be interesting to see how the neoadjuvant or adjuvant administration of more aggressive chemotherapy regimens such as S1 [16] and FOLFIRINOX [31–33] and advances in radiation oncology will affect incidence, timing, and pattern of PDAC recurrence. Translational studies characterizing the molecular properties of PDAC in the context of the pattern of disease may identify biomarkers associated with systemic progression that may become useful for personalized decision-making in the management of PDAC recurrence.

Surveillance After Resection for Pancreatic Cancer

The effectiveness of surveillance after PDAC resection is highly controversial, and in most countries structured surveillance programs are not established. While some of the available treatment guidelines for PDAC do not even address this relevant topic, several "evidence-based" guidelines give out different recommendations with respect to follow-up after potentially curative surgery (Table 2). Based on very similar literature, the German S3 guidelines do not recommend structured surveillance programs for PDAC due to a lack of evidence of positive effects of surveillance on prolonging survival after the completion of adjuvant chemotherapy [22], while the North American NCCN guidelines acknowledge the lack of evidence but still recommend CA 19-9 examination and cross-sectional imaging every 3–6 months for the first 2 years. The latter recommendation was based on the consensus that earlier

detection of recurrence may facilitate patient eligibility for investigational studies or other forms of treatment [21]. However, a cost-effectiveness analysis revealed higher costs without any survival benefit from a regular follow-up program that included abdominal imaging [34].

On the one hand, it should be acknowledged that clinical guidelines have to be based on current evidence and have to include socioeconomic considerations and that there is at present little evidence for the benefit of surveillance. On the other hand, structured surveillance programs are needed to enable studies investigating the potential survival benefit from early detection and timely therapy of PDAC recurrence.

Although regular surveillance is not generally recommended and usually not paid by the health insurances, some centers offer a structured follow-up with physical examination, blood tests (including CA 19-9 levels), and abdominal imaging to all patients who undergo PDAC resection. In a recent analysis of 940 postoperative follow-up visits performed in 618 pancreatic patients over a 1-year period, recurrences were detected in 74 (40%) of 184 patients in follow-up after PDAC resection, of whom only 26% had symptoms [35]. In all of these patients, a cancer-directed therapy was initiated. Importantly, 12 (75%) of 16 patients with isolated local recurrence were without symptoms and 11 were referred for re-resection [35]. The comparison of sequential follow-up CT scans allows for early detection of local recurrences by identification of subtle but progressive changes at typical predilection sites for local recurrences (Fig. 1) [36]. The value of CT scans in the early detection of local recurrence was recently confirmed in an independent series [37].

These data have important implications, because they show that most recurrences are at first asymptomatic and will be detected earlier with regular surveillance including cross-sectional imaging, and this offers the opportunity for earlier initiation of cancer-directed therapy. While it appears logical that earlier detection of recurrence and initiation of therapy may result in better outcomes, future studies will have to assess how the treatment options discussed below affect survival and quality of life of patients with PDAC recurrence.

The development of tools for screening of risk populations and for early detection of PDAC is an area of intensive research. Novel analytic targets such as exosomal markers and cell-free DNA that are currently being evaluated for early detection of PDAC may also be promising tools for post-resection surveillance [38, 39]. The potential of structured surveillance programs after resection will have to be redefined in the future as better diagnostic tools, and more effective systemic therapies will hopefully become available.

Treatment of Recurrence of Pancreatic Cancer

The treatment of pancreatic cancer recurrence is based on very limited evidence. The available literature is restricted to mostly small retrospective studies in selected patients and/or multiple case reports, suggesting a considerable publication bias.

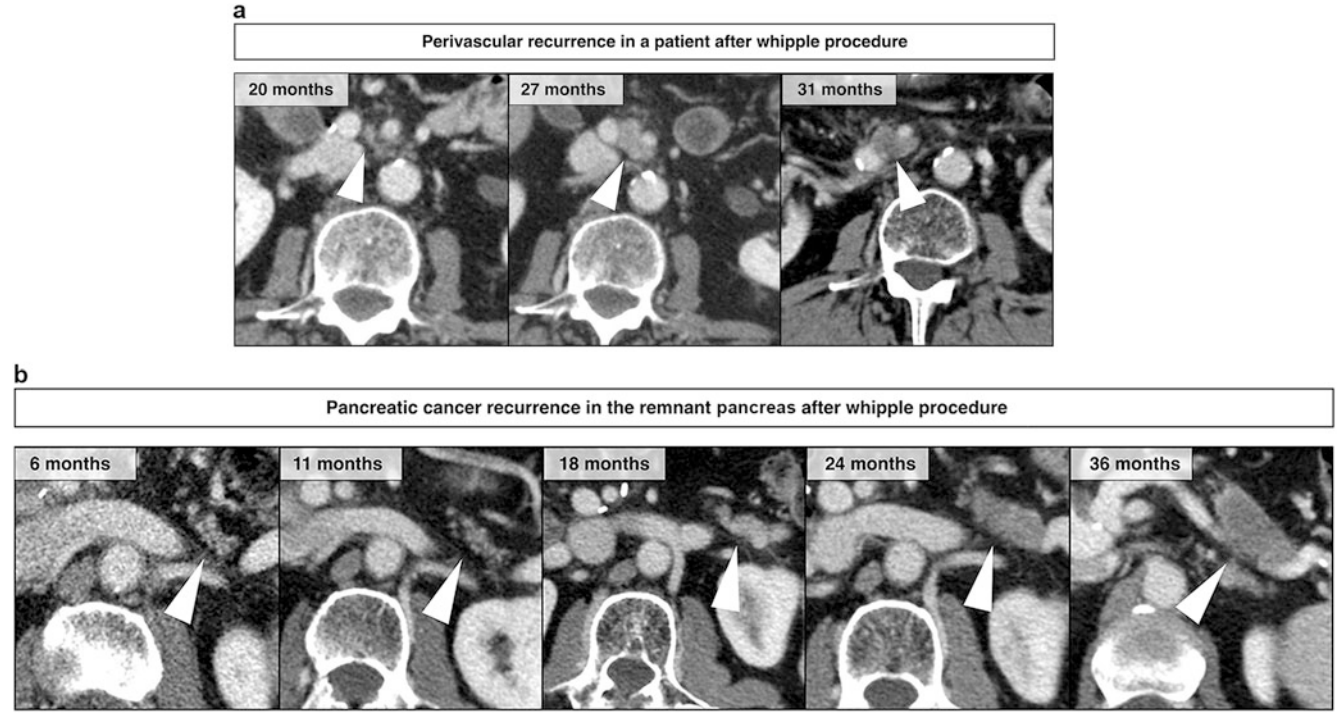

Fig. 1 Typical findings of local recurrence in sequential CT scans for surveillance after resection for pancreatic adenocarcinoma. (**a**) Perivascular recurrence at different time points after pancreatoduodenectomy for pT3pN1R1 ductal adenocarcinoma in a 64-year-old patient. Unsuspicious findings at 20 months. At 27 months increase of dense tissue between the superior mesenteric artery and vein with further increase and hypodense changes after 31 months. The patient underwent successful re-resection with intraoperative radiation therapy (*IORT*). (**b**) Local tumor recurrence involving the pancreatic remnant after pancreatoduodenectomy for pT3N1R1 ductal adenocarcinoma. Unsuspicious findings until 18 months after resection. Hypodens changes in the pancreatic remnant at 24 months with increase after 36 months. The patient underwent re-resection with completion pancreatectomy and adrenalectomy

While several of the current treatment guidelines do not even address the topic of treatment of PDAC recurrence, the German S3 [22] and the NCCN guidelines [21] mention several treatment options dependent on the pattern of recurrence including local therapy for local recurrences (Table 3) [21–26]. Based on common sense rather than on actual evidence, the pattern of recurrence defines the potential benefit of additional local versus merely systemic treatment. The appropriate treatment options further depend on multiple parameters including the exact localization of recurrence, the clinical performance status and comorbidity of the patient, previous cancer-directed treatment (i.e., neoadjuvant and/or adjuvant treatment regimens), and timing of recurrence (i.e., interval between resection and recurrence and timing in relation to adjuvant therapy).

Available treatment options described in the literature are summarized in Table 4 in the context of the pattern of recurrence The following paragraphs address treatment options for systemic recurrence and isolated local recurrence separately.

Treatment of Systemic Recurrence

As discussed above, the majority of patients with recurrence after PDAC resection present with systemic disease. Clearly, systemic chemotherapy is the appropriate cancer-directed therapy for the majority of these patients. There is little evidence from the literature as to the best regimen in this situation. However, this is a palliative situation, and depending on the timing of recurrence (during or after adjuvant therapy), the regimen used for neoadjuvant and/or adjuvant therapy, and the performance status of the patients, the same principles as outlined for second-line chemotherapy in advanced disease and for palliative treatment may be recommended (see chapters ▶ "Palliative Management of Pancreatic Cancer" and ▶ "Chemotherapy for Advanced Pancreatic Cancer"). Among current treatment guidelines, the NCCN guidelines provide the most detailed recommendations adjusted to the possible clinical scenarios (Table 4) [21]. With respect to quality of life, adequate pain therapy, management of cancer complications, and supportive care are very important aspects of palliative therapy in patients with PDAC recurrence (see chapter ▶ "Palliative Management of Pancreatic Cancer").

Oligometastatic Recurrence

None of the current guidelines specifically address the situation of oligometastatic recurrence of PDAC in their main recommendations, because the evidence on the management of this condition is limited to small case series and case reports of oligometastatic recurrence in the liver and lungs. Metastasectomy for both initially systemic disease and systemic PDAC recurrence is highly controversial. However, recent reports suggest that such operations are increasingly performed [40–43]. Data on resection of metachronous liver metastases is limited to case reports and subgroup analyses of small series. The few available series on resection for liver metastases of PDAC mainly analyze synchronous resection and resection in patients with good response to chemotherapy, and the median survival of 14–15 months is not very

Table 3 Recommendations on treatment of recurrent pancreatic cancer in selected recent clinical guidelines

Guideline	Recommendation	Level of recommendation	Level of evidence
AWMF Germany (2013) [22]	*7.13*:	GCP – strong consensus	NA
	Local recurrence: In case of isolated local recurrence for pancreatic cancer, all possibilities for local therapy should be considered		
	Systemic recurrence: Not specifically addressed		
NCCN USA (2016) [21]	*MS-44/PANC-10*:	Category 2B	Lower level
	Confirmatory biopsy		
	All cases of recurrent disease	Category 2A	
	➔ Clinical trial is preferred option	Based upon lower-level evidence, there is uniform NCCN consensus that the intervention is appropriate	
	➔ Palliative and best supportive care without additional therapy should also be an option		
	Local recurrence:		
	Chemoradiation can be considered in patients with local disease recurrence only, if not previously administered		
	An alternative chemotherapy regimen can be given		
	Surgical resection may be considered in select cases (i.e., good performance status, location of recurrence is favorable), though there is currently no evidence to support this recommendation		
	Systemic recurrence:		
	<6 months after adjuvant therapy → alternative chemotherapy		
	6 months after adjuvant therapy → systemic therapy as previously administered or an alternative systemic regimen		
	Previous adjuvant treatment and good performance status → gemcitabine/nab-paclitaxel and FOLFIRINOX		
ASCO USA (2016) [23]	Not addressed	NA	NA

(*continued*)

Table 3 (continued)

Guideline	Recommendation	Level of recommendation	Level of evidence
ESMO Europe (2016) [24]	Not addressed	NA	NA
NCI USA (2016) [25]	**Local recurrence**: Not addressed	NA	NA
	Systemic recurrence:		
	Palliative chemotherapy		
	Chemotherapy: fluorouracil or gemcitabine		
	Treatment options under clinical evaluation (refers to clinical trials)		
IAP and EPC consensus review of guidelines (2015) [26]	Not addressed	NA	NA

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encouraging [40, 41]. In 23 patients who underwent resection for metachronous metastases of PDAC, the median survival after diagnosis of liver metastases was 14.5 months (unpublished data) in line with the published series.

In contrast, favorable survival has consistently been reported after resection of the rare event of isolated lung metastases including metastatic pulmonary recurrences [42, 44–47]. PDAC with isolated pulmonary metastases appears to identify a subgroup with favorable prognosis, probably explained by unique molecular properties of these tumors [43, 48, 49]. The favorable survival observed after resection of pulmonary metastases may, thus, at least in part be explained by a selection bias.

While there is no evidence for a survival benefit of local therapies for metastatic disease, metastasectomy may be considered in selected patients with oligometastatic hepatic and particularly pulmonary recurrences, especially in patients with good performance status and a long interval between resection and diagnosis of the metastatic recurrence. Other local treatment options such as locally ablative therapies or radiation therapy may also be considered.

Treatment of Isolated Local Recurrence

The evidence on treatment of isolated local recurrence of PDAC is limited, but there are promising results from several retrospective cohort studies or case series for multimodal concepts including chemoradiation and surgical re-resection (Tables 5 and 6) [46, 50–61]. The use of local ablation therapies such as irreversible

Table 4 Cancer-directed treatment options for recurrent pancreatic cancer

Type of recurrence	Possible treatment	Intention	Comments
Isolated local recurrence	Chemotherapy	Palliation	Considered the standard therapy for any kind of recurrence without curative intention
			Data based on cohort studies
	Chemoradiation	Palliation/ local control/ pain therapy	Considered by many as only alternative treatment option that includes a local therapy
			Data based on cohort studies (see Table 5)
	Re-resection in combination with chemotherapy or chemoradiation	Potential cure/medium- to long-term control	Re-resection in a multimodal setting in combination with chemotherapy/chemoradiation is the only potentially curative treatment option. Performed in highly specialized surgical centers
			Data based on cohort studies (see Table 6)
	Locally ablative therapies in combination with chemotherapy	Palliation/ local control	Experimental treatment options (including irreversible electroporation, radiofrequency ablation, etc.). Data mainly extrapolated from cohort studies in unresectable disease. Only case reports in the setting of isolated local recurrence
Oligometastatic systemic recurrence	Chemotherapy	Palliation	Standard treatment for metastatic recurrence
			Data based on cohort studies in recurrence. Preferred regimens mainly extrapolated from studies on second-line treatments for primarily unresectable/metastatic disease
	Metastasectomy in combination with chemotherapy	Medium- to long-term control	May be appropriate for selected patients
			Limited data from small retrospective cohort studies. Best data for pulmonary metastases
	Locally ablative therapies in combination with chemotherapy	Medium- to long-term control	May be appropriate for selected patients
			Data restricted to case reports

(*continued*)

Table 4 (continued)

Type of recurrence	Possible treatment	Intention	Comments
Systemic recurrence	Chemotherapy	Palliation	Standard treatment for metastatic recurrence
			Data based on cohort studies in recurrence. Preferred regimens mainly extrapolated from data on second-line treatments for primarily unresectable/ metastatic disease

Table 5 Retrospective series of radiation therapy for local recurrence of pancreatic cancer

Author	Year	N included	Radiotherapy	Chemotherapy	Oncologic outcome
Wilkowski [50]	2006	18	45 Gy	5-FU ($n = 4$)	OS: 17.5 months
				5-FU, Gem ($n = 6$)	PFS: 14.7 months
				Cis, Gem ($n = 8$)	CR: $n = 6$ (33%)
Wild [51]	2013	18[a]	SBRT 25 (20–27) Gy	28% ($n = 5$)	OS: 8.8 months
Habermehl [52]	2013	41	39.6–54 Gy + IORT (15 Gy) in $n = 15$	Gem (90%)	OS: 16.1 months
				5-FU or Cap (10%)	PFS: 6.9 months
					CR: $n = 6$ (15%)
Nakamura [53]	2014	30	54 (39–60) Gy	Gem ($n = 18$)	OS: 15.9 months
				S1 ($n = 7$)	PFS: 6.9 months
Zeng [54]	2016	24 ($n = 5$ additional metastases)	SBRT 45 (42–50) Gy	Reported in $n = 3$	OS: 12.2. months
					PFS: NA
					CR: $n = 5$ (21%)

Included are studies with >5 patients undergoing chemoradiation
SBRT stereotactic body radiation therapy, *OS* overall survival, *PFS* progression-free survival, *CR* complete response (clinical). Updated from Strobel and Büchler [17]
[a]Study includes $n = 3$ patients after definitive chemoradiation (no resection) for locally advanced disease

electroporation and radiofrequency ablation may represent another strategy worth testing for isolated local recurrences (Table 4). However, as data on local ablation therapies are restricted to case reports, the following paragraphs will focus on radiation therapy and surgical re-resection.

Only two of the analyzed guidelines specifically address the treatment of isolated local recurrence (Table 3). The current German S3 guidelines recommend the evaluation of available local therapies and mention the options of re-resection and chemoradiation in the supporting discussion [22]. The NCCN guidelines are more specific and recommend first inclusion in clinical trials (preferred), the administration of chemoradiation (if not previously done), a change of the regimen of systemic chemotherapy, or palliative and best supportive care. In their 2/2016 version, the NCCN guidelines for the first time mention the option of surgical re-resection in the supporting discussion, but continue with the statement that "there is currently no evidence to support this recommendation" [21].

Given the available data on radiation therapy and re-resection discussed below and summarized in Tables 5 and 6, this preference of chemoradiation over re-resection is somewhat startling and may point to a certain dominance of radio-oncologists in the guideline panels.

Rational for Local Therapy

Undisputedly, most pancreatic cancer patients will eventually die from metastatic spread even after potentially curative resection. However, it has been generally accepted that in primary pancreatic cancer, surgical resection in combination with systemic chemotherapy (or chemoradiation) is currently the only therapy option offering long-term survival and, in rare cases, even cure [2].

With advances in both safety and radicality, the limits of surgical resection are today being pushed toward extended resections [62] or resections after aggressive neoadjuvant therapies for locally advanced PDAC [31, 32] with promising results. Strategies of neoadjuvant treatment offer the advantage to select patients without progression for surgical resection, while patients with early systemic progression are selected out. Similarly, an isolated local recurrence may identify patients with tumors of a less aggressive phenotype resulting in slower systemic progression and better prognosis [29, 57]. This notion provides a good rational to test localized treatments such as re-resection and chemoradiation in this selected subgroup of patients with a localized disease pattern. However, the majority of patients presenting with suspected isolated local recurrence may also have occult systemic disease and may develop systemic progression later in the course of their disease. Therefore, as for primary pancreatic cancer, local therapies for recurrence must always be embedded in multimodal treatment strategies that include systemic chemotherapy.

Radiation Therapy for Isolated Local Recurrence

Radiation therapy/chemoradiation is often discussed as the main alternative to merely palliative chemotherapy for treatment of local recurrence [21]. The evidence for chemoradiation is based on only few retrospective series of limited size (Table 5) [50–54]. The actuarial overall median survival reported for different radiation therapy protocols ranges between 8.8 and 17.5 months. The three series using chemoradiation report longer median survival around 15.9–17.6 [50, 52, 53] compared to the two series on stereotactic body radiation therapy (SBRT, 8.8 and 12.2 months) [51, 54], probably because fewer patients received additional systemic

Table 6 Retrospective series of re-resection for local recurrence of pancreatic cancer

Author	Year	N operated	N resected	Resection rate	Mortality	Other cancer-directed therapy	Oncologic outcome
Kleeff[a] [55]	2007	30	15	50%	1 (7%)	Chemoradiation: $n = 7$	Re-resection:
						Radiation: $n = 1$	OS: 17.0 months
						Chemotherapy: $n = 9$	Exploration:
						2nd re-resection: $n = 6$	OS: 9.4 months
						None: $n = 4$	
						NA: $n = 9$	
Lavu [56]	2011	NA	8	NA	0	NA	OS: 17.5 months
Thomas[b] [46]	2012	NA	7	NA	0	NA[b]	OS: NA[b]
							DFS: 9 months
Strobel[a] [57]	2013	97	41	42.3%	1 (1.8%)	In cases with re-resection:	Re-resection:
						Chemoradiation: $n = 22$	OS: 26.0 months
						IORT: $n = 22$	
						Chemotherapy: $n = 21$	Exploration (ILR):
						NA: $n = 4$	OS: 10.8 months
							Exploration (M1):
							OS: 9.4 months

Boone[b] [58]	2014	NA	10	NA	0	NA	OS: 31.8 months
Miyazaki [59]	2014	NA	11	NA	0	Chemotherapy: $n = 8$	Re-resection:
						None: $n = 3$	OS: 25.0 months
							No re-resection:
							OS: 9.3 months
Shima [60]	2015	NA	6	NA	0	Chemotherapy: $n = 1$	OS: 27.5 months
Chang [61]	2016	NA	7 PDAC	NA	0	NA	Re-resection:
							OS: 8.9 months
							Exploration:
							OS: 5.8 months

Included are studies with >5 patients undergoing re-resection

OS overall survival, *DFS* disease-free survival, *ILR* isolated local recurrence without evidence of systemic disease confirmed by surgical exploration. Updated from Strobel and Büchler [17]

[a]The cohort by Kleeff et al. is from the same center and included in the follow-up study by Strobel et al.

[b]Mixed cohort of local and distant recurrence. Only data of patients with local recurrence included here

chemotherapy in the latter two studies. Data on progression-free survival, local control rates, toxicity, and symptom relief are all inconsistently reported among the available studies. It should be noted that three studies report complete radiologic response rates of 15–33% [50, 52, 54].

With clear evidence for local efficacy in all studies and overall survival rates of up to 18 months, treatment strategies that include radiation therapy to improve local control should be further tested in patients with isolated local recurrence after resection of PDAC. However, the data also suggest that local radiation has to be accompanied by systemic chemotherapy to achieve adequate progression-free and overall survival.

Re-resection for Isolated Local Recurrence

Very similar to the situation described for radiotherapy, the evidence for re-resection for isolated local PDAC recurrence is based on retrospective series of limited sample size (Table 6) [46, 55–61]. However, the reported outcome with median overall survival rates of 25 to >30 months after re-resection in four of the more recent series [57–60] is superior to the outcome reported after chemoradiation. These differences can in part be explained by bias due to the exclusion of patients with radiologically undetectable metastatic disease in the resected subgroups. While most series did not report on resection rates, the series from Heidelberg initially reported a resection rate of 50%, which dropped to 42.3% in the larger follow-up study, mainly due to intraoperative diagnosis of metastases [55, 57]. Overall, the available series clearly show that re-resection for isolated local recurrence is feasible and safe (low mortality rates of 0–2%) and associated with encouraging survival results. However, it should be emphasized that these results are based on cohorts of highly selected patients treated in specialized referral centers for pancreatic surgery and may not be commonly applicable.

The initial experience with re-resection at Heidelberg University Hospital was reported in 2007 [55], and the so far largest series on re-resection for isolated local PDAC recurrence was published in 2013 [57]. Of 97 patients with preoperatively suspected isolated local recurrence and histologic proof of recurrence, 57 (59%) had isolated local recurrence by surgical exploration, while distant metastases were identified in 40 (41%) patients. This highlights the necessity of better diagnostic tools to detect small metastatic deposits, a problem known from staging of primary PDAC. Of 57 isolated local recurrences, 41 (72%) were resected (Fig. 2), while 16 (28%) were locally unresectable. Median postoperative survival was 16.4 months in confirmed isolated local recurrence versus 9.4 months in metastatic recurrence, confirming the better prognosis associated with localized disease pattern observed in other studies [63]. Importantly, median survival in isolated local recurrence was significantly longer after re-resection compared to locally unresectable recurrences (26.0 vs. 10.8 months). This observation in surgically confirmed isolated local recurrence clearly points to a potential survival benefit from re-resection. R0 re-resection in 18 patients was associated with a favorable median survival of

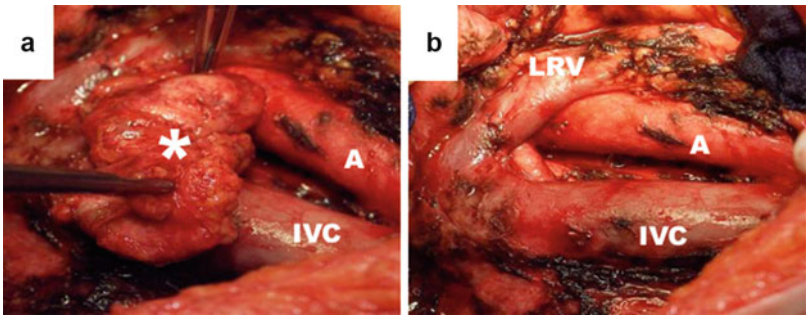

Fig. 2 Intraoperative findings in a patient with isolated locoregional recurrence in a typical predilection site after resection for adenocarcinoma of the pancreatic head, interaortocaval lymph nodes below the left renal vein. (**a**) Location of the recurrence (*) after exposure and dissection. (**b**) Operative site after tumor removal and retroperitoneal lymphadenectomy. *A* abdominal aorta, *IVC* inferior caval vein, *LRV* left renal vein

30.5 months [57]. While a true benefit of re-resection can only be demonstrated by RCTs, these results suggest that selected patients with suspected isolated local PDAC recurrence may benefit from re-resection.

Only one study [61] that did not report on administration of systemic therapy reports a sobering median survival of 8.9 months after re-resection for PDAC recurrence. This again points toward the need to embed re-resection in a multimodal treatment strategy that includes systemic chemotherapy in order to achieve long-term survival.

Overall, the available series on re-resection clearly demonstrate that this concept is promising and should be tested in selected patients. A direct comparison of survival outcomes reported for radiation therapy and re-resection is not scientifically sound and should not be made. However, it is very clear that the available evidence for re-resection is at least equal, if not superior, to the evidence on radiation therapy in terms of the numbers and sample size of studies as well as with respect to reported survival outcomes.

Selection of Patients for Local Therapy

The identification and selection of patients that benefit from a treatment that includes local therapy are very relevant in the context of PDAC recurrence. Clearly, patients with a localized disease pattern without systemic progression, in whom the local recurrence may define prognosis, are the most likely to benefit from local therapy.

To identify these patients, early detection of the local recurrence by adequate surveillance and a thorough diagnostic workup to minimize the risk of occult metastatic disease are necessary. However, as discussed above, the rate of undetected metastatic disease is high [57]. While PET-CT is a currently available technology which holds promise in detection of local and distant PDAC recurrence and warrants

further investigation [64], better tools for detection of metastatic disease are needed in the future.

The larger available series on treatment of local PDAC recurrence analyzed parameters that are associated with survival and may be useful for patient selection. The interval between primary tumor resection and detection of recurrence [46, 53, 55, 58] and CA 19-9 serum levels [57] are two parameters that may be useful, but the available data do not yet allow to determine cutoff values to support decision-making. In the absence of clear evidence, patients with a long interval between primary tumor resection and detection of local recurrence, low tumor markers, good performance status, and low comorbidity are probably the best candidates for local therapies based on common sense.

There is accumulating evidence that molecular properties of the primary tumor define the pattern of localized versus metastatic disease and even the distribution of metastatic disease between organs (e.g., liver and lung) [29, 48, 49]. Recently, several distinct molecular subtypes of pancreatic cancer that are associated with treatment response and prognosis have been identified [65–68]. Similar studies may allow for identification of molecular signatures associated with localized disease or systemic progression and serve as new powerful tools for patient selection in the future.

Conclusions

Pancreatic cancer recurrence is a pressing problem that affects the vast majority of patients even after successful resection and completion of adjuvant chemotherapy. It is, therefore, surprising how little evidence there is with respect to the management of pancreatic cancer recurrence, and it is concerning that this important topic is still missing in many current clinical practice guidelines for pancreatic cancer. While the majority of patients develop metastatic recurrence, a significant subgroup of 20–30% of patients first develop isolated local recurrence. These patients appear to have tumors of less aggressive subtypes with slower systemic progression and may benefit from local therapy. As most recurrences are at first asymptomatic, structured follow-up programs are needed for earlier detection and timely initiation of therapy. However, in the absence of evidence, structured surveillance programs are currently not recommended. Although the literature provides little evidence with respect to the management of isolated PDAC recurrence, both chemoradiation and surgical re-resection appear to be safe and effective based on several retrospective series. The best "standard" management for isolated PDAC recurrence can only be determined based on RCTs which are unlikely to be conducted for this indication. More likely, the therapy for PDAC recurrence will remain a matter of interdisciplinary, personalized decision-making. Novel biomarkers for early detection of PDAC and the development of more effective systemic treatments will hopefully also advance surveillance after PDAC resection and treatment of PDAC recurrence.

Cross-References

- ▶ Adjuvant Chemotherapy in Pancreatic Cancer
- ▶ Chemotherapy for Advanced Pancreatic Cancer
- ▶ Neoadjuvant Chemotherapy in Pancreatic Cancer
- ▶ Palliative Management of Pancreatic Cancer

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Management of Cystic Neoplasms of the Pancreas Including IPMNs

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Abstract

The management of cystic pancreatic lesions fundamentally depends on knowing the cyst type and the risk or presence of malignancy. Only serous cystic neoplasms (SCN) are generally benign lesions, while mucinous cystic neoplasms (MCN) and intraductal papillary mucinous neoplasms (IPMN) as the most common cystic lesion and solid-pseudopapillary neoplasm (SPN) show different risk profiles for the development of invasive cancer. Once a cystic lesion is detected, the clinical decision is necessary if an upfront resection with the inherent morbidity of pancreatic surgery should be performed or if an observational management can be preferred. Whereas these strategies are clearly defined for certain cystic lesions including SCN, MCN, and SPN, the management of IPMN, especially with regard to the branch-duct type, remains partly controversial, and current guidelines differ with regard to indications for surgery and/or surveillance. The present chapter gives an overview on the different types of pancreatic cystic neoplasms and current diagnostic modalities. Furthermore, the indications for surgery, the variety of surgical resections, and the surveillance/follow-up strategies are discussed in the light of the current literature and guidelines.

Keywords

Intraductal papillary mucinous neoplasm · Mucinous cystic neoplasm · Serous cystic neoplasm · Solid-pseudopapillary neoplasm

Abbreviations

AGA	American Gastroenterological Association
BD	Branch duct
CDX	Caudal-related homeobox transcription factor
CT	Computer tomography
ERCP	Endoscopic retrograde cholangiopancreaticography
EUS	Endoscopic ultrasound
FNA	Fine needle aspiration
IAP	International Association of Pancreatology
IPMN	Intraductal papillary mucinous neoplasm
KRAS	Kirsten rat sarcoma viral oncogene homolog
MCN	Mucinous cystic neoplasm
MD	Main duct
MRCP	Magnetic resonance cholangiopancreaticography
MRI	Magnetic resonance imaging
MUC	Mucin protein
NECP	Neuroendocrine cyst of the pancreas
PanIN	Pancreatic intraepithelial neoplasm
PDAC	Pancreatic ductal adenocarcinoma
SCN	Serous cystic neoplasm
SPN	Solid-pseudopapillary neoplasm
VHL	von Hippel-Lindau

Introduction

For pancreatic cysts, an overall prevalence of 2.5% is estimated in recent reports from the United States. In MRI studies, they may be seen in 14–20% up to 50–70% in people aged >70 years [1, 2]. A former postmortem study describes pancreatic cysts <1 cm in app 25% of cases [3]. Most of them are asymptomatic incidental findings, more and more detected by increased use of improved imaging. The meaning of these features depends on the potential of the different types to develop malignancy. Arising awareness leads to a worldwide discussion in literature and expert meetings with lots of effort to acquire guidelines concerning diagnostics, risk factors, course, and management of each form of pancreatic cystic lesions, guided by symptoms and risk of malignancy.

In contrast to real cystic lesions of the pancreas, pseudocysts should be sharply distinguished. Pseudocysts can mostly be found as sequela of inflammation or trauma and are easy to differentiate and to diagnose. Due to their size and their almost extrapancreatic appearance, pseudocysts were frequent findings also in times of moderate accuracy of imaging, and therefore it formerly was assumed that most cystic lesions of the pancreas were pseudocysts. Their clinical relevancies being benign residual lesions concern only symptoms and signs of inflammation to indicate any form of therapy. Therefore, they will not be subject of this chapter.

Under consideration of the wide range of different cystic neoplasms, the most frequently resected and clinically relevant entities include intraductal papillary mucinous neoplasm (IPMN), mucinous cystic neoplasm (MCN), serous cystic neoplasm (SCN), and solid-pseudopapillary neoplasm (SPN). In large collectives of patients who underwent resections for cystic pancreatic lesions ($n > 400$, from the Memorial Sloan Kettering Cancer Center New York and >800 from Harvard, Boston), IPMN was documented in 23–38%, MCN in 11–23%, SCN in 16–23%, and SPN in 2–3% [4, 5]. They differ in incidence, localization, and age and sex correlation and show specific gene alterations. Only SCN is considered to be benign, while IPMN, MCN, and SPN show different malignant potentials, which are associated with their mucinous components. Different types of IPMN can be found in the Wirsung or Santorini duct (main duct, MD-IPMN) as well as in the branch ducts of the pancreas (BD-IPMN) and are of high interest for recent research due to their risk to become pancreatic cancer which can be estimated at 70% and 25–30%, respectively, for MD- and BD-IPMN.

This chapter gives an overview to the current knowledge of diagnostics and behavior of the different cystic lesions of the pancreas, which is the basis for understanding their relevance and for developing individual therapy strategies. The first part comprises the knowledge for each type of cystic pancreatic neoplasms under consideration of large patient series. Diagnostic procedures are summarized and discussed within the second part with respect to the accuracy of preoperative diagnostics, as the key tool for further management. In the third section, the controversial possibilities and opinions on how to handle pancreatic cystic lesions once they are detected – surveillance versus resection – are highlighted under consideration of recent guidelines.

Characterization of the Different Types of Pancreatic Cysts

Dilated Branch Ducts

Dilation of branch ducts of the pancreas is a very common incidental finding in CT or MRI scans with increasing prevalence. Seventeen percent of all people show these changes with increasing age [2, 6]. The terminology of BD dilation describes all visible cystic BD lesions of <10 mm in diameter. They are not associated with any abdominal symptoms, and annual control by MRI or EUS is adequate (see Sect. 4). Their role as precursors to BD-IPMN and the natural course in terms of growth dynamics are not totally understood yet. Despite this currently incomplete understanding, they are also described as "incipient IPMN" by a recent and new histological definition [7]. Once their diameter exceeds 10 mm, these lesions fulfill imaging criteria of BD-IPMN.

Intraductal Papillary Mucinous Neoplasms

Intraductal papillary mucinous neoplasms (IPMN) account for approximately 35% of all cystic pancreatic tumors and consequently represent the largest subgroup. IPMNs are characterized by production of mucin as well as intraductal and papillary growth of the ductal epithelium. With regard to their location in the pancreatic duct system, they are subclassified into main-duct (MD), branch-duct (BD), or mixed-type IPMN, involving both the main duct and the side branches [4, 7]. To date, it remains controversial whether mixed-type IPMNs primarily arise from the main pancreatic duct and from side branches or if both structures are simultaneously affected, and they therefore represent a distinct subtype of IPMN. IPMNs have to be clearly differentiated from pancreatic intraepithelial neoplasms (PanINs) as another precursor lesion to pancreatic ductal adenocarcinoma (PDAC).

MD- and mixed-type IPMNs are characterized by a dilation of the main pancreatic duct >5 mm without any sign of an external obstruction, which can be found only segmentally or diffusely. The neoplastic papillary epithelium produces abundant mucin with a high viscosity which cannot be drained sufficiently and leads to obstruction and secondary dilation of the affected parts of the duct system. BD-IPMNs are defined as cysts >10 mm communicating with the pancreatic main duct without its dilation (Fig. 1) [8]. Although most IPMNs are primarily noninvasive, they show a potential for a malignant transformation over time following an "adenoma-carcinoma" sequence via three or four grades (low-grade, (formerly also borderline), high-grade dysplasia, and invasive cancer [7].

Four main aspects characterize the natural history of IPMN patients:

- Morphological type (MD-, BD- or mixed-type IPMN)
- Age at the time of diagnosis and the time course of the disease
- Histological subtype (intestinal, pancreatobiliary, oncocytic, gastric differentiation)
- Grade of dysplasia (low-grade, borderline, high-grade dysplasia, invasive cancer)

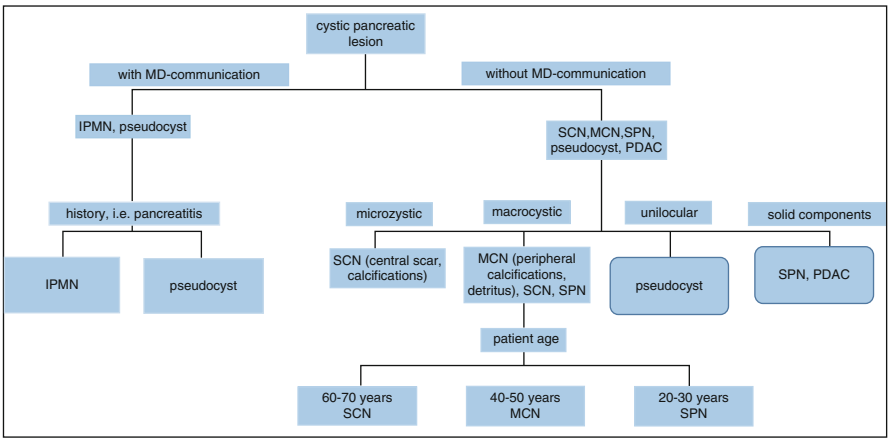

Fig. 1 Radiological flowchart for the diagnostic algorithm of cystic pancreatic lesions (Adopted from [6])

Morphological Type (MD-, BD- or Mixed-Type IPMN)

A recent and comprehensive meta-analysis on the incidence of malignancy in IPMN has shown a rate of 43% for invasive cancer in MD- and mixed-type IPMNs, whereas this is found in app. 17% of BD-IPMNs [9]. In addition to true invasive cancer, lesions with high-grade dysplasia are also regarded as "malignant" by many authors due to the consideration that high-grade dysplasia reflects a situation where there is no more time to waste as the inherent progression to invasive cancer may occur very soon [10]. Consequently, including high-grade dysplasia lesions, MD-IPMNs show an overall risk of malignancy of 60%, whereas BD-IPMNs show malignant transformation in 20–25% of all cases. Mixed-type IPMNs seem to be associated with the highest risk of malignancy which is estimated to be app. 70% in large study populations [8]. The dynamic and time frame of progression for the different morphological IPMN types is not completely understood yet, as especially for MD- and mixed-type IPMN – which are basically resected by the time of diagnosis – no reliable data are available. Furthermore, it has to be considered that radiologically defined findings of BD-IPMN may contain a mixed-type component in the histological workup when they are resected. This underlines the difficulty to evaluate an individual patient's risk on the basis of morphological characteristics.

Patient Age at the Time of Diagnosis

The median age of patients presenting with benign IPMNs compared to malignant IPMNs shows significant differences in two cohort studies of 140 resected MD- and mixed-duct IPMNs by Salvia et al. [11] as well as in 136 resected MD-IPMNs by Sohn et al. [12]. From both studies, the progression to invasive IPMN can be estimated at 5–6 years as patients with benign IPMNs showed a median age of 61 and 63 years, compared to 67 and 68 years for patients with malignant findings [11, 12]. This estimation can certainly only be regarded as a surrogate parameter, and an

already longer subclinical course of preceding IPMN development by the time of diagnosis must be considered. Another corresponding observation is the correlation between duct diameter and the risk of malignancy in MD-IPMNs, underlining that a potentially longer course of the disease reflected by the increased duct size leads to a higher proportion of malignant findings [13].

Progression rates of BD-IPMN under observation have been reported in large studies by Sahora et al. [14] and Maguchi et al. [15]. Among 411 resp. 349 patients, signs of progression occurred in a proportion of 18% during a median follow-up time of 26 months and 44 months, respectively. Malignant histological features after resection of these patients were finally found in 9% and 15% of the patients. Furthermore, both studies demonstrated the development of "remote" lesions – both IPMN and PDAC – distant from the index lesion during the observation. This underlines that the phenomenon of IPMN may not be a focal and localized defect, but the entire pancreas may be affected by a genetic "field defect" with a disparate penetrance in different regions of the gland. This hypothesis is also supported by the synchronous occurrence of multiple BD-IPMNs observed in a certain proportion of patients [16]. Patients with multifocal lesions are generally older than those with solitary IPMN findings, and although multiple lesions are likely to increase the long-term risk of malignant transformation, it remains controversially debated whether multifocal IPMNs have a higher risk of malignancy compared to unifocal lesions [16]. The additional 10-year risk for IPMN patients to develop concomitant PDAC can be estimated between 3% and 9% [17]. If IPMN might play a promoting role in the development of PDAC remains unclear.

Histological Subtypes

Four histological IPMN subtypes with a relevant prognostic impact can be differentiated, namely, intestinal, pancreatobiliary, oncocytic, and gastric subtype. The *intestinal* subtype which displays malignant features in app. 50% of all cases is characterized by neoplastic epithelial cells expressing MUC2, MUC5AC, and CDX2 as typical markers and is mainly found in MD-IPMN [17]. Invasive cancers arising from intestinal-type IPMN are usually colloidal carcinomas and show a better median survival compared to PDAC (107 vs. 20 months) [18]. The *pancreatobiliary* subtype shows branched papillary epithelia with high-grade atypia and an immunohistochemical positivity for MUC1 and MUC5AC. Ninety percent of all IPMNs of this subtype show an associated invasive component, typically tubular adenocarcinomas, which is the most aggressive IPMN-associated cancer and very similar to PDAC in morphology and prognosis [19]. The *oncocytic* subtype is characterized by eosinophil cytoplasm, goblet cells, and complex branched papillary epithelia expressing MUC1 and MUC6. This subtype is rare as well as a malignant transformation into an oncocytic carcinoma which shows a prognosis similar to patients with a colloid carcinoma [19]. BD-IPMNs usually show a *gastric* subtype morphology with multiple small cysts with foveolar gland epithelium, resembling glands of the gastric antrum. A tubular adenocarcinoma can eventually arise from these IPMNs and is associated with an intermediate prognosis with a mean survival of only 45 months [20].

Grade of Dysplasia

Patients after resection of any type of noninvasive IPMNs (only low-grade and high-grade dysplasia according to the recent Baltimore guidelines [7]) show an excellent overall and disease-specific 10-years survival of 95–100% for both MD- and BD-IPMN [17]. In invasive IPMN, poor prognosis is closely related to disease stage, positive resection margins, and N1 status [17, 21]. Early stages of IPMN-associated cancer including pT1 and pN0 show a much more favorable prognosis compared to sporadic PDAC. However, once advanced stages (pT2–pT4) and especially lymph node metastases are found, survival decreases significantly, and the prognosis is not superior to sporadic PDAC [21].

Mucinous Cystic Neoplasms

Mucinous cystic neoplasms (MCNs) are typically found in perimenopausal women with a median age of 48 years and are often located in the distal body or the tail of the pancreas (>90%) with a mean diameter of 6 cm at the time of diagnosis. MCNs show a uni- or multicystic pattern with a thick wall and can display solid components. The important differentiation to BD-IPMN is the lack of any communication to the pancreatic duct system. The typical and pathognomonic histopathological finding is an ovarian-like stroma in these lesions [22].

Similar to IPMNs, most MCNs are noninvasive but show a risk of an "adenoma-carcinoma" sequence over time. In larger series on resected MCNs, in 15–20% of all cases an invasive component is found [23]. Since patients with an invasive MCN are significantly older (median 3–10 years) than those with a noninvasive MCN, a time-dependent tumor progression is likely, comparable to that in IPMN [8]. Although the impact of the ectopic ovarian stroma in MCN remains unclear, a hormone- and growth factor-dependent induction with a consecutive progression from pancreatic epithelium to cystic lesions is discussed. This potential correlation is supported by the observation of a rapid growth of pancreatic MCNs in women during pregnancy. Features of potential malignancy in MCN are mural nodules on imaging, lesion size >6 cm, and calcification of the cyst wall [24]. Once an MCN is diagnosed, a surgical resection is indicated in most cases due to the young age of the patients and the present inability to differentiate securely between a benign and a malign lesion. The 5-year overall survival of patients presenting with invasive MCN is app. 60%, being worse for elderly patients and for patients with more advanced tumor stages [25].

Serous Cystic Neoplasms

Serous cystic neoplasms (SCNs) are mostly found in the pancreatic body and tail and lack a significant potential for malignant transformation. The incidence of SCNs is slightly higher in women than in men with a peak at the age of 60 years. In cross-sectional imaging and in resection specimen, SCNs have a micro- or macrocystic appearance with a typical finding of a central scar structure. Histopathologically,

they are composed of cysts lined by a single layer of glycogen-rich cuboid epithelial cells [26]. A preoperative distinction of SCN from MCN is possible in most cases due to significant differences in imaging. Besides sporadic SCNs, which represent the most common entity, there is an association with von Hippel-Lindau syndrome in some patients. In von Hippel-Lindau patients, SCNs are commonly multiple, whereas sporadic SCNs are mostly single lesions. Sporadic SCNs have a somatic mutation of the VHL gene in up to 50% with an inactivation of the VHL tumor suppressor protein [26–28] and often show a mutation in the TBC1D3 gene, also known as PRC17, but no mutations in the genes typically mutated in mucinous neoplasms, such as KRAS, RNF43, or TP53 [28, 29].

At the time of diagnosis, SCNs have a mean size of 4–6 cm and half of the patients are asymptomatic [26]. Depending on the localization and size of the lesion, symptoms including abdominal pain, discomfort, jaundice, or fatigue may occur. Malignant transformation of SCN leading to a serous cystic adenocarcinoma is very rare and has only been described in few case reports [30]. Thus, for asymptomatic patients with an SCN of <4 cm in diameter and without criteria for malignancy on preoperative imaging, only surveillance is indicated. The natural course of SCN is characterized by a gradual increase in diameter (0.6 cm/year in average). The growth rate seems to be size depending as small SCNs (<4 cm) show a significantly slower growth rate of 1–2 mm/year than larger lesions (>4 cm) in which annual growth rates of up to 2 cm can be found. Consequently, besides the size itself, growth rate during surveillance may have an influence on the decision for surgery to avoid local complications due to compression. Following resection, recurrence risk is extremely low and no structured follow-up is recommended [31].

Solid-Pseudopapillary Neoplasm (Frantz Tumor)

Initially described in 1959, solid-pseudopapillary neoplasms (SPNs, Frantz tumors) are rare cystic neoplasms and account for approximately 1–2% of all pancreatic tumors [32]. They are usually found in young women with a median age of 30 years and are most frequently located in the tail of the pancreas. SPNs show the potential of lymphatic spread, recurrence, and distant metastases and are therefore classified as malignant lesions [33]. Nearly all reported series on SPN include surgical patients who underwent resection, and nonsurgical management has been described only anecdotally. Consequently, the natural history of SPN in terms of growth dynamics and malignant progression remains unclear. Although long-term survival with locally limited tumor manifestation seems possible, also aggressive systemic spread with short survival times are found underlining the malignant potential of *SPNs*.

SPNs have a mean size of 8 cm by the time of diagnosis and are mainly (60%) located in the body and tail of the pancreas [33]. Their macroscopic appearance shows a combination of solid and cystic components, and nearly all tumors show characteristic mutations in exon 3 of the β-catenin gene [34]. A specific absence of other common mutations, such as KRAS, SMAD4, or TP53, distinguishes SPNs from other neoplasms of the pancreas [35].

The long-term prognosis of SPN after resection is excellent although app. 6% of the patients show locally advanced tumors with vascular involvement or lymph node metastases and 8% present with distant metastases [33]. A recent review including more than 2 200 resected patients shows that 96% of the patients are disease-free during long-term observation. The time to recurrence in the remaining 4% of the patients is more than 4 years, and the overall disease-specific mortality is 1.5% [33]. Despite this general favorable prognosis, SPN are basically malignant tumors and complete surgical resection is indicated as well as a lifelong follow-up (i.e., annually) [32].

Diagnostic Modalities

In general, pancreatic cysts are classified as either nonneoplastic or neoplastic. Nonneoplastic cysts include pseudocysts, retention cysts, and benign epithelial or lymphoepithelial cysts and are not associated with any tendency for progression to malignancy, whereas in neoplastic cysts, the differentiation between serous and mucinous lesions is essential to evaluate the risk of malignant transformation. Mucinous cysts are more common and harbor a certain risk of malignancy, depending on various criteria which include type of lesion and size as well as other features specified in detail for every entity above.

Diagnostic Modalities 1: The Radiological View

Contrast-enhanced CT scan and MRI are the preferable cross-sectional imaging modalities for the clarification of cystic neoplasms of the pancreas. As MRI offers a very good visualization of fluid and soft tissues, this modality is superior in showing septation, debris, and nodules and often allows a more specific diagnosis than CT. For demonstrating a communication of the cyst with the pancreatic ductal system, magnetic resonance cholangiopancreaticography (MRCP) is the imaging tool of choice. In cases of difficulties in detecting the communication, secretin given during the MRCP may be helpful [6].

Beyond visualization of the pancreatic duct system, this specific advantage of MRI, CT, and MRI is equally effective. Moreover, CT is superior in detection of calcification of pancreatic cysts compared to MRI [36] and offers the advantages of lower costs and broad availability. However, it has to be considered that radiation exposure associated with repeated CT examinations limits its suitability for long-term surveillance of cystic lesions. Therefore, when frequent imaging is required, this exposure can be avoided by using MRI with MRCP.

The first step in the radiological differentiation of a pancreas cyst in MRI is to estimate its communication with the main pancreatic duct, which is the precondition for distinguishing an IPMN from other cystic lesions. Only pseudocysts are to consider as a differential diagnosis, communicating also sometimes with the pancreatic ductal system [37].

Secondly, the radiologist has to proof the morphology of the pancreatic cysts [6]:

- *Unilocular cysts* show no septs or solid portions; this feature is mostly seen in pseudocysts and rarely in SCN, MCN, and IPMN.
- *Microcystic lesions* show single small cysts <2 cm and are mostly found in SCN or BD-IPMN.
- *Macrocystic lesions* consist of less but bigger compartments (>2 cm) than microcystic lesions. Normally, they can be demonstrated in MCN or IPMN and rarely in neuroendocrine tumors or lymphangioma.
- Cysts with *solid contents* are often found in SPN, but also in MCN and IPMN or degenerative altered cysts. Solid contents are generally suspicious of a malignant potential.
- *Calcifications* located in the middle of a cyst are suggestive of SCN, while calcifications built like peripheral eggshells are specific for MCN.

The third aspect in radiologic diagnostic is to consider the cyst's localization within the pancreas combined with certain clinical characteristics including age, sex, history of pancreatitis, and elevation of laboratory blood values, which should be taken into account. SCNs are often found in elderly women ("grandmother tumor"). Macrocystic lesions in the corpus or tail of the pancreas in fertile women often turn out to be MCN ("mother tumor"), while macrocystic lesions in the pancreatic head of an elder man are highly suspicious to be an IPMN. In contrast, SPNs are typical for young women ("daughter tumor") [33]. For unilocular cysts, first of all an underlying pancreatitis should be excluded via history and blood analysis [6].

MD-IPMNs are mainly not presenting "classical" cystic features from the radiological point of view but are diagnosed by imaging as a dilation of the MPD ≥5 mm, either segmental or diffuse, without identifiable reason for an external pancreatic duct obstruction and without signs of pancreatic branch-duct dilation. If combined with one or more dilated branch ducts (≥10 mm), they fulfill the criteria of a mixed-type IPMN.

For the differentiation between benign and potentially malignant IPMN and therefore the management decision (resection vs. surveillance), specific radiologic criteria have to be considered, which were initially defined in the IAP consensus guidelines in 2006 [8] and have been updated in the following Fukuoka meeting in 2012 [9] (Table 1). Figure 1 gives an overview of the diagnostic radiological modalities.

Diagnostic Modalities 2: The Endoscopic View

Endoscopic ultrasound (EUS) is a well-established examination modality, which allows a transluminal high-resolution diagnostic examination of the pancreatic parenchyma and the ductal system. Evaluation of a pancreatic mass or pancreatic cyst is the most common indication for EUS of the upper gastrointestinal tract [38]. As the differential diagnosis of cystic lesions which are asymptomatic often requires

Table 1 Radiologic criteria for clinical decision-making on how to manage BD-IPMN and estimation of their malignant potential [9]

Worrisome features	High-risk stigmata
Non-enhancing mural nodes	Enhancing solid components
Main-duct diameter 5–9 mm	Main-duct diameter ≥10 mm
Abrupt change in main-duct caliber with distal parenchyma atrophy	
Thickened/enhancing cyst wall	
Cyst size ≥3 cm	

additional diagnostic tools after an initial cross-sectional imaging (CT scan or MRI), EUS offers a cost-effective approach to decide whether surgery is warranted or radiologic and clinical surveillance can be recommended [39].

EUS is particularly valuable in evaluating diagnostic features and potential risk factors for malignancy as it has been shown to have a high sensitivity and specificity for these questions, including size and number of cysts, thick vs. thin cyst wall, nodules, septa, solid contents, diameter of the main pancreatic duct, and its communication with the cyst as far as the presence of lymph nodes [40]. In general, EUS is not superior but comparable to MRI for identifying main-duct involvement and the communication with the cyst and for detecting mural nodes missed in CT/MRI. Furthermore, EUS offers the possibility of fine needle aspiration (FNA) and characterization of a pancreatic cystic lesion by obtaining cyst fluid analysis, which may be helpful for clinical decision-making. The fluid can be evaluated for tumor markers, as well as cytopathologic, biochemical, and molecular analysis. For the tumor marker **CEA**, the initial study by Brugge et al. could demonstrate that an optimal cutoff value of 192 mg/mL is associated with a diagnostic accuracy of 79% for detection of mucinous cysts and the differentiation from serous – and consequently harmless – cysts [41]. Recent analyses studying the value of CEA in cyst fluid calculated a positive predictive value of 96% for CEA levels greater than 400 ng/ml and a negative predictive value of 98% for CEA levels below 5 ng/ml [42]. To note, the level of CEA in cyst fluid does not correlate with malignancy but may only be used for the characterization of a mucinous nature of the cystic lesion [43]. In contrast, carbohydrate antigen (CA) 19-9 does not have any significant predictive value in the diagnosis of a mucinous lesion [44].

Fluid cytology can detect malignant cells, MUC-containing cells (IPMN and MCN mentioned above), glycogen-rich cuboidal cells (SCN), branching papillae with myxoid stroma (SPN), and abundant anucleate squamous cells and debris (lymphoepithelial cysts) [38]. The accuracy for the detection of mucinous cysts resp. malignancy is shown to be 58% resp. 75%.The analysis of cyst fluid **DNA** is often performed for detection of KRAS mutation, which is highly specific for a mucinous cyst (96%) [44].

In general, the use of fluid cytology is not regarded as a standard to date, regardless if the cystic lesion shows worrisome imaging features or not [45]. According to several reports, the sensitivity in determining malignancy in pancreatic cysts using EUS-FNA is 50% or even less [42]. The abovementioned limitations in the currently available analysis of cyst fluid underline the need for improved diagnostic tools. Recent studies could demonstrate the potential impact of **novel molecular markers**, including VEGF, GNAS, mi-RNA, mucic stain, or inflammatory mediator proteins [46, 47]. Consequently, the value of analysis of cyst fluids retrieved by FNA is currently still in a preclinical stage for the safe determination of pre-malignancy or malignancy but a specific field of ongoing intense research [40].

A further aspect is the option of an EUS-guided therapy with **cyst ablation**, which may provide a minimally invasive alternative to surgery in patients not suitable for an operation. First results on small patient collectives show complete cyst resolution in <40% of patients using ethanol installation, also in long-term follow-up, and cyst resolution with minimal residuum of the cyst in app. 60–80% in patients after injection of paclitaxel [40]. However, this procedure should not be considered as an alternative to surgery as it is still unclear concerning the effects on the natural history of cysts and the long-term outcomes. It should therefore only be performed after critical evaluation in individual cases when patients are not suitable candidates for a surgical approach.

Moreover, to date not every center can provide EUS facilities which limit its widespread use. Another potential shortcoming of EUS is the lack of reproducibility and the high dependency of quality and results on the expertise of the examining physician. Considering these aspects, EUS – with or without FNA – is not suitable as an exclusive but as an additional diagnostic tool as it may improve the diagnostic accuracy of cross-sectional imaging for pancreatic cystic lesions. Also ERCP is not utilized as a routine examination tool for the differentiation of pancreatic cystic lesions [43, 48]. In some cases, mucous secretion into the duodenum, highly suspicious for MD-IPMN, can be seen by ERCP or esophagogastroscopy [43].

Diagnostic Modalities 3: The Pathologic View, Including Genetic Aspects

The first step to differentiate pancreatic cysts after their resection is the distinction in neoplastic and nonneoplastic (congenital, lymphoepithelial, enterogene, endometrial, lymphangioma, hemangioma, sarcoma) as far as epithelial and non-epithelial (pseudocysts and parasitic cysts). All pancreatic cysts mentioned in this chapter are neoplastic and of epithelial origin [49]. The epithelial cells differ in appearance (i.e., columnar in IPMN and MCN, cuboidal in SCN) and express different glycoproteins (several types of the so-called MUC). They produce an either serous or mucinous cyst fluid, the latter associated with a higher risk for malignancy. Further immunohistopathologic parameters possibly expressed by the different cyst epithelia include CEA, α-inhibin, neurospecific enolase (NSE), caudal homeobox protein (CDX)-2,

Table 2 Histomorphological and genetic patterns of different pancreatic cysts (Adopted from [49])

Pancreatic cyst	Histopathology	Immunoprofile	Genetics	Differential diagnosis
IPMN	Mucin-producing epithelium with typical cystic dilation of the pancreatic ducts	MUC expression:	Mutations of KRAS, GNAS (intestine IPMN), RNF 43	MCN
		Gastric type: MUC5		
		Intestine type: MUC 2 + 5, CDX2		
		Pancreatic type: MUC 1 + 5		
		Oncocytic type: MUC 1, 2, 5, 6		
MCN	Mucin-producing epithelium, "ovarian" stroma	*Epithelium*: CEA + MUC5 + *Stroma:* progesterone and estrogen receptor+, a-inhibine+		IPMN
SCN	Multicystic low epithelium	*Serous epithelium*: MUC1+, MUC6+ α-inhibine +		NCC metastasis, BD-IPMN
SPN	Eosinophil epithelium, hyaline stroma	Vimentin + CD10+ progesterone receptor + β-catenin nuclear +	Mutation CTNNB1-gene (exon 3)	NET, acinar cell carcinoma

vimentin, CD10, and β-catenin. In addition, the cyst stroma appearance between the cells of the pancreatic cysts widely differs, i.e., ovarian-like in MCN and hyaline in SPN. Considering genetic aspects, KRAS, GNAS, and RNF43 mutations are common pathological findings (Table 2).

IPMN in the general pathologic view is defined as *a grossly visible*, *predominantly papillary*, *or – more rarely – flat*, *noninvasive mucin-producing epithelial neoplasm arising in the main pancreatic duct or branch ducts*. Macroscopically – as ideally in the radiological imagings already described – they are found intraductal either affecting the main pancreatic duct or the branch ducts. For further differentiation of IPMN, four microscopic **subtypes** are of interest: the *gastric type* is found in BD-IPMN [20], while the largest proportion (36%) of MD-IPMNs shows an *intestinal type* (36%). A *pancreatobiliary* resp. *oncocytic* subtype accounts for 7–8% of all IPMN each [19]. The term **incipient IPMN** describes branch-duct lesions between 0.5 and 1.0 cm in diameter with intestinal or oncocytic differentiation or with a GNAS mutations, which typically occur in intestinal and gastric IPMN subtypes [7]. Furthermore, the **grade of dysplasia** is an important topic in the pathologic examination with respect to therapy and prognosis. It represents the epithelial changes on the way to malignancy of IPMN and MCN, which are well-characterized in terms of an adenoma-carcinoma sequence and are reflected by an increasing number of genetic alterations. Concerning recent guidelines for both IPMN and MCN, low-grade dysplasia has to be differentiated from high-grade

dysplasia and invasive cancer [7]. In some centers, still the former classification including borderline between low- and high-grade dysplasia is used.

Accuracy of Preoperative Diagnostics According to the Definitive Histopathologic Result

The management of pancreatic cysts fundamentally depends on knowing the cyst type and the risk or presence of malignancy. This underlines the importance of a correct diagnosis at the time of detection. Salvia et al. could show an accuracy of 78% for preoperative diagnosis of any cystic pancreatic lesion in 476 resected patients when they matched preoperative and final pathologic diagnosis in a retrospective approach [50]. The best results were achieved for SPN (95%) and for IPMN with main-duct involvement (81%). EUS showed no additional diagnostic benefit. In another series including 334 patients, IPMN with main-duct involvement was correctly diagnosed in the preoperative cross-sectional imaging in 71% [52]. Jang et al. compared the results of preoperative CT, MRI, and EUS findings in 318 Korean patients with the final pathology after resection of pancreatic cysts [53]. The sensitivity to predict the type of pancreatic cysts was 83% vs. 94% and 89% for CT alone vs. CT and additional MRI or EUS, and the specificity was 70% vs. 59% and 53%, respectively. The diagnostic accuracy of a combination of CT and MRI (81%) was superior to CT alone (61%) and EUS (70%). In distinguishing mucinous from non-mucinous cysts in a multicenter trial of 341 patients, EUS accuracy was only 51% [41], while other authors describe an accuracy up to 73% [50–54]. **In conclusion**, MRI is the tool of choice for the differentiation and diagnosis of cystic pancreatic lesions, preferable to CT, and potentially supplemented by EUS with or without FNA.

Management of Pancreatic Cystic Lesions

As described for each cystic entity above, the malignant potential is essential for the further clinical decision with regard to surveillance or an upfront operation as well as for surveillance and follow-up intervals and duration, respectively. Furthermore, the malignant potential needs to be considered with regard to the extent of resection if an operation is indicated. Other important aspects in the decision-making process concerning an operation are age and comorbidity of the patient, and no prophylactic resection is indicated in patients with a higher risk of perioperative life-threating complications than for experiencing the malignant transformation of their pancreatic cyst to PDAC. Consequently, although general indications for surgery in specific cystic lesions exist, individual decisions are possible, which is reflected in all current guidelines as recommendations always refer to “patients who qualify for surgery” or “patients fit for surgery” [31, 55].

Option 1: Operation

Indications for Surgery

Surgery is indicated in SCN only if patients are symptomatic, the lesion exceeds 4 cm in diameter, or there is a clear progression with an annual growth of >6 mm [30].

In contrast, MCN resection is basically indicated by the time of diagnosis, independently of symptoms or size [9, 31]. Although a current publication including a large collective of 349 MCN patients challenges this general recommendation [56, 57], international guidelines are not adopted yet, and the indication for resection seems to be unquestionable to date [57].

A similar general recommendation for resection is given for all MD- and mixed-type IPMNs with a main pancreatic duct diameter of ≥10 mm [9, 31]. A recent study, showing that MD- and mixed-type IPMNs with a duct diameter below 10 mm bear a significant risk of malignancy as well [58], raises the question if the threshold for resection should potentially be lowered in updated guidelines. However, this remains a point of controversy to date.

The most controversial current aspect is the indication for and the timing of resection in BD-IPMN. Based on the 2012 consensus guidelines [9], the so-called "Sendai" criteria have been established to describe the risk of malignancy in these lesions. The guidelines recommend the resection of branch-duct IPMN of more than 3 cm in diameter in general. Smaller branch-duct IPMN should only be resected in the presence of "high-risk" stigmata including mural nodules, positive cytology, symptoms, or a synchronously dilated main duct. However, there is growing evidence that these guidelines are not sufficient enough in order to recognize all premalignant lesions in time. In different larger surgical series examining resected IPMN, the incidence of malignant branch-duct IPMN (including in situ and invasive carcinoma) was approximately 25% among all IPMN below 3 cm without any reliable cutoff in diameter [10, 59, 60] (Table 3). Although these are certainly selected collectives of patients, the findings of malignant potential in a relevant proportion of the patients underline that a clear stratification and decision for conservative or surgical treatment is very difficult up to the present. Neither the existence of mural nodules as a guideline predictor of malignancy nor the existence of clinical symptoms did correlate with malignancy. These findings underline that size alone and currently established markers of potential malignancy are not reliable predictors and that even small branch-duct IPMNs have a relevant risk of malignancy. Individual decisions for resection based on an evaluation of all morphological and clinical factors (including imaging, tumor markers, symptoms, progression, and prior patient history) seem to offer the best approach at the moment.

Finally, for all SPN, there is an agreement that a surgical resection is indicated by the time of diagnosis, regardless of any additional symptoms or associated findings [31].

Table 3 Reported rates of malignancy in various series of small branch-duct IPMN in retrospective surgical collectives

Study	n	Malignancy rate (high-grade dysplasia or invasive cancer)			
		<1 cm	1–2 cm	2–3 cm	Total <3 cm
Schmidt et al. 2007 [59]	103	3/18	8/53 (16%)	5/29 (17%)	16/82 (20%)
Jang et al. 2008 [60]	138	1/31 (3%)	7/42 (17%)	6/25 (24%)	14/89 (16%)
Walsh et al. 2008 [75]	56	–	–	–	12/56 (21%)
Fritz et al. 2012 [10]	123	3/12 (25%)	11/40 (28%)	3/17 (18%)	17/69 (25%)
Wong et al. 2012 [76]	105	4/7 (57%)	5/19 (26%)	31/44 (70%)	40/70 (57%)
Sahora et al. 2013 [14]	217	0/4 (0%)	6/46 (13%)	15/75 (20%)	21/125 (17%)

Types of Surgical Resection

Formal Resections

Standard procedures for surgery of cystic lesions include partial, distal, and total pancreatectomy [61]. These operations can be performed for any type of cystic entity (SCN, MCN, IPMN, SPN), depending on the localization and size of the lesion. For findings limited to the pancreatic head, pylorus-preserving pancreatoduodenectomy is the routine approach. A classical pancreatoduodenectomy with stomach resection is rarely required and should be restricted to situations where the lesion extends toward the pylorus and gastric antrum. Preservation of the pylorus offers the advantage of physiological food passage and is therefore regarded as superior regarding weight loss and quality of life in the long-term outcome, which may be especially important for patients resected for benign pancreatic lesions with a good prognosis. In case of suspected MD-IPMN, after completion of the resection, it is mandatory to perform an examination of the pancreatic resection margin by intraoperative frozen section. The surgical strategy has to be adjusted afterwards. In case of IPMN-free cut margins, no further resection is required. In contrast, when IPMN manifestations are found at the site of transection, this implies that completion pancreatectomy should be considered, depending on various factors. These include the grade of dysplasia at the transection site, the localization on IPMN spread in the parenchyma or in the main pancreatic duct, the age of the patient, and the finding in the resected pancreatic head. In case of IPMN-associated invasive cancer in the resected specimen, an individual decision has to be made as the prognosis is determined by this invasive component and is not dependent on the remaining IPMN tissue which implies that the pancreatic remnant may be preserved. An oncological lymphadenectomy should always accompany formal resections of main-duct IPMN and SPN according to their malignant potential. This comprises the lymph nodes of the hepatoduodenal ligament as well as the lymph nodes along the right side of the celiac axis and the superior mesenteric artery. The reconstruction includes pancreaticojejunostomy or pancreaticogastrostomy, hepaticojejunostomy, and duodeno- or gastrojejunostomy.

If the cyst is located in the pancreatic body or tail, distal pancreatectomy is the standard resection [61, 62]. For MD-IPMN and SPN, this operation is again performed following oncological principles, including lymphadenectomy along the left side of the celiac axis, the superior mesenteric artery, and the hepatoduodenal ligament as well as splenectomy. In case of benign IPMN or MCN, spleen preservation is possible, either with or without preservation of the splenic vessels.

Division of the pancreas above the portal vein/superior mesenteric vein axis can be done by stapling devices or scalpel followed by suture closure of the remnant. Coverage of the resection margin by patches (e.g., jejunum/teres hepatis ligament, artificial patches) or a pancreaticojejunostomy to avoid postoperative pancreatic fistula is optional. Especially coverage by a teres ligament flap has the potential to reduce associated clinical complications [63]; however, none of the mentioned methods has yet been proven to actually decrease the overall POPF incidence, which ranges between 30% and 50%.

Total pancreatectomy is required for diffuse main-duct IPMN or – rarely – for extended manifestations of multifocal BD-IPMNs. It is performed either as a primary en bloc resection if the IPMN extension is preoperatively assessed throughout the entire gland or as a sequential procedure in situations where intraoperative frozen sections show IPMN progression after partial pancreatectomy, as described above. A splenectomy and lymphadenectomy combining the lymph node regions of partial pancreaticoduodenectomy and distal pancreatectomy are required, as total pancreatectomy should also be carried out oncologically.

Parenchyma-Sparing Resections

Parenchyma-sparing resections comprise enucleation and central pancreatectomy. Enucleation is a suitable approach for small (<3 cm) cystic lesions that are located in a subcapsular position and show an adequate (3 mm) distance to the main pancreatic duct. This distance is essential as it has been shown that "deep" enucleation for lesions with a distance of less than 3 mm to the pancreatic duct is associated with a significantly higher risk for POPF and should be evaluated carefully against the possibility of a formal resection [64]. Predominantly, enucleation is feasible for SCN and BD-IPMN. It can be performed if the benign character of the excised lesion is confirmed by intraoperative frozen section and when the location and morphology of the cystic lesion are suitable for this procedure. In order to evaluate this adequately, an accurate localization of the cystic lesion is essential. Besides preoperative imaging, the most important tool for tumor location is the experience of the surgeon performing the exploration [65, 66]. Mobilization of the pancreas and a careful digital examination of the suspected lesion are supplemented by intraoperative ultrasound examination if necessary. By means of intraoperative ultrasound, not only an identification of the cystic lesion is feasible but moreover the relation and distance to the pancreatic duct can only be clarified [65]. During enucleation itself, careful attention needs to be paid to the connection of the cyst to the pancreatic duct.

This should be identified and closed by clip or suture ligation to avoid high-volume enzyme leakage. A tumor size of 3 cm in diameter can be regarded as the limit for a safely performed enucleation. Tumors measuring more than 3 cm in size show malignant histological changes significantly more often, making a local surgical approach impossible. Besides, tissue trauma and wound surface following an enucleation reach a critical size for the development of fistulas or other complications, including bleeding or postoperative pancreatitis. The resected cyst should always be examined by intraoperative frozen section to confirm its benign nature. In the case of unexpected malignancy, a more extended oncological resection must be chosen. Drain placement at the end of the operation is recommended as fistula rates of approximately 30% are currently reported; however, most of them are clinically irrelevant [65, 66].

The second limited and parenchyma-sparing resection approach for localized and benign pancreatic cysts located in the body of the pancreas is central pancreatectomy. A segment between the level of the superior mesenteric vein/portal vein axis and the remaining tail of the gland can be resected under preservation of all healthy tissue [67, 68]. Pancreatic transection toward the pancreatic head is performed similar to distal pancreatectomy, mostly by stapler or by scalpel with a consequent suture closure. Toward the pancreatic tail, the transection is performed in a way, comparable to partial pancreaticoduodenectomy, mostly by sharply to avoid tissue damage on the cut margin. After removing the cyst-bearing segment, the distal stump of the pancreas is further mobilized from the splenic vessels over a 2 cm distance to allow a safe anastomosis. Reconstruction is accomplished with a retrocolic Roux-en-Y loop of the jejunum. Alternatively, a pancreaticogastrostomy is possible. The already closed pancreatic head remnant can finally be covered with the same jejunal loop by sutures between the seromuscular layer of the jejunum and the capsule of the pancreas. Another possibility to reduce clinically relevant POPF-associated complications is the use of a ligamentum teres flap for covering of the stump, which has been shown to be beneficial in distal pancreatectomy and can be used in central pancreatectomy as well [63].

Reconstruction is completed by an infracolic Roux-en-Y enteroenterostomy in case of pancreaticojejunostomy as the method of reconstruction [68]. To date, fistula rates of approximately 40% are reported for central pancreatectomy. Comparable to enucleation, most of these fistulas are uncomplicated, do not lead to consecutive complications, and can be treated conservatively [67, 68].

Parenchyma-sparing resections have been described for MCN and SPN in the past in several series. As MCN is comparable to BD-IPMN in terms of malignancy risk, these procedures are a suitable possibility for this entity, presumed that the benign character of the lesion is confirmed intraoperatively. In contrast, for SPN, non-oncological resections have to be evaluated critically, as the nature of the lesion cannot be predicted in most cases, and the impact of lymph node dissection remains unclear. Due to the small reported patient numbers, valid data on this topic are not available to date.

Laparoscopic Surgery

Laparoscopic pancreatic surgery has become increasingly important during the last decade. Cystic lesions are findings that specifically qualify for this approach, as the procedure is technically not burdened by peripancreatic tissue alterations which are commonly found in chronic pancreatitis or pancreatic cancer, but are not present in cystic lesions. Laparoscopic distal pancreatectomy is the most commonly performed procedure for cystic neoplasms today. Although no randomized controlled trials have shown superiority of laparoscopic distal pancreatectomy compared to the open approach, the minimally invasive procedure is regarded as a standard of care in most centers, especially for benign indications. The extent of resection in the laparoscopic setting is similar to the open procedure with regard to lymphadenectomy and splenectomy vs. splenic preservation depending on the dignity of the removed cystic lesion.

Besides distal pancreatectomy, enucleation and central pancreatectomy are infrequently performed laparoscopically; the available literature demonstrates the technical feasibility and perioperative safety of both – laparoscopic enucleation and central pancreatectomy – but is dominated by case series data and most commonly limited to 5–30 patients [69–71].

Option 2: Surveillance

In general, all pancreatic cysts should be discussed in multidisciplinary boards in specialized centers for pancreatic diseases. Currently, it could be shown in a large survey in the Netherlands that despite of varying guidelines, the risk of malignancy is underestimated by a significant proportion of physicians, and a majority suggests abdominal ultrasound as an adequate surveillance tool in cysts of 10 mm [72]. These results may reflect a substantial lack of awareness for the malignant potential of IPMN, MCN, and SPN. Especially in the United States, the recent guidelines of the AGA [55] have induced an important and controversial discussion about the surveillance management, as the recommendations do not match those of other international guidelines [31, 73], and the statements of a reduced or even no surveillance for asymptomatic small cyst under consideration of the health care costs are not in accordance with long-term results of various studies showing an ongoing and potentially increasing risk of malignancy, even beyond a 5-year period.

As pointed out above, only asymptomatic SCN <4 cm and some BD-IPMNs show no indication for an upfront operation. For these entities, structured lifelong surveillance is recommended in 6 monthly up to annual intervals, depending on the nature and course of the cysts. As the time from the first diagnosis of an MD-IPMN to development of an invasive IPMN can be estimated at 5–6 years [11, 12], for BD-IPMN, no data exist, but progression in 18% of BD-IPMN patients during a median follow-up time of 26 months and 44 months was observed [14, 15], indicating the

high likelihood in an unknown part of BD-IPMNs for a slow development of malignancy with unknown point of no return. Thus, especially for middle-aged patients, even shorter surveillance intervals (6 monthly) are discussed initially after 5 years, during those one observation visit per year could be sufficient. In case of SCN, besides the size itself, the growth rate triggers the decision for surgery to avoid complications caused by local compression.

Every surveillance visit should cover MRI/MRCP, alternatively EUS in experienced hands, a physical examination, and a blood analysis, containing routine parameters and HbA1c, CEA, and CA 19-9. Moreover, the individual history of pain or even pancreatitis must be considered. In any case of deterioration – if new pancreas-related symptoms, new-onset diabetes, weight loss, increase of the tumor markers without any other cause, increasing of cyst size, or other changes in imaging as radiologic criteria of possible malignancy (newly detected worrisome features or high-risk stigmata according to [8]) are observed – an operation has to be evaluated.

For patients who are not fit for surgery at the time of diagnosis of a pancreatic cystic neoplasm without signs of malignancy but an indication for resection, a surveillance strategy should be chosen, which is adapted to the specific physical and psychosocial condition of each individual patient. In case of a progress of the lesion and if a suspicion of malignancy occurs, a biopsy and histopathological workup are required. In case of a confirmation of invasive cancer, chemotherapy with or without radiation should be discussed depending on the physical performance status of the patient.

Postoperative Follow-Up

As all types of IPMN as far as MCN and SPN must be considered as a chronic and lifelong disease – unless a total pancreatectomy has been performed – the natural course of these entities requires regular postoperative follow-up regarding to international guidelines [9, 31, 55]. Lately, published follow-up data from surgical IPMN patients showed that 17% of 381 patients after resection of invasive and noninvasive IPMN had a recurrence of the IPMN after a median of 17 months [74]. Within this study, 33 patients had only partial resection of the multifocal disease with mixed-type as well as BD-IPMN. The residual BD-IPMNs with a median size of 10 mm at the date of resection grew within a follow-up of median 5 years to a median size of 13 mm. In another cohort of 130 patients who had undergone partial pancreatic resections for noninvasive IPMNs, He et al. showed that 17% of the patients developed lesions suspicious for new or progressive IPMN within a median time of 46 months [73]. Within this disease progression cohort, some patients developed high-grade dysplasia and invasive cancer. Another 12% of the cohort showed neither new IPMN nor progression in known residual IPMNs. Although within the literature the recurrence rates vary between 8% and 57% [11, 44, 73], even patients with noninvasive IPMN might have an estimated average recurrence rate of 25% of remote IPMN and 7% for developing pancreatic cancer within 5 years after resection. For MCN, the risk for recurrence seems to be lower than for IPMN as

noninvasive MCNs show no recurrences after complete resection and do not require a structured postoperative surveillance [9, 31]. In contrast, follow-up after resection of invasive MCN should be performed similar to PDAC [31].

After resection of SPN, a recurrence rate of 4 up to 11% is described with a mean time to recurrence of 1 to >4 years [30, 72, 73]; therefore, a long-term follow-up 1–2×/year is recommended [31, 73].

Comparable to the recommendations for surveillance of cystic lesions without primary operation indication shown in Table 4, postoperative follow-up visits for noninvasive pancreatic cysts should also include MRI (alternatively by endosonographic ultrasound in experienced hands), physical examination, and blood analysis. Also the loss of function after pancreas resection is considered in this postoperative setting. In addition for IPMN patients, regular endoscopic controls focused on colorectal adenomas and Barrett dysplasia of the esophagus are recommended as both pathologies are increasingly observed in IPMN patients [20, 21]. The recommended intervals per cyst entity are shown in Table 4.

In conclusion, recent results underline the necessity of a structured and long-term follow-up after resection of every pancreatic cyst, except for SCN. In the case of confirmed recurrence, surgical re-resection should be attempted according to the recommendations given above. Depending on the extent of the prior resection, this implies the performance of a remnant pancreatectomy in a considerable number of patients.

Table 4 Proposal of a management algorithm for cystic pancreatic lesions and postoperative follow-up: time intervals and imaging modalities [9, 31, 74, 77]

		Proceeding (time interval in months)		Postop. follow-up (time interval in months)
Diagnosis		Surveillance (MRI/EUS)	Resection	MRI (CT)
Serous cystic neoplasm (SCN)	<4 cm, asymptomatic	6, 18, 30, . . .annually		
	Symptomatic or >4 cm		x	None
Mucinous cystic neoplasm (MCN)			x	None
Dilated branch ducts		12, 24, 36, . . .annually		
BD-IPMN	Without worrisome features*	12, 24, 36, . . .annually		
	With worrisome features*		x	MRI: 6, 18, 30, . . .annually
MD-IPMN			x	MRI: 6, 12, 18, 24, . . .6 monthly
Mixed-type IPMN			x	MRI: 6, 12, 18, 24, . . .6 monthly

*see table 1

Conclusion

Pancreatic cysts are common entities and are increasingly found due to improved imaging modalities. As they bear a certain risk of malignancy, the indication for surgery has to be evaluated by the time of diagnosis; however, not all cystic lesions require a surgical intervention. While there are clear recommendations with regard to the management of SCN, MCN, and SPN, clinical decision-making in IPMN remains controversial. The current knowledge on the risk of malignancy in IPMN is mostly based on retrospective surgical series although recently an increasing number of publications deal with the natural course of IPMNs under a watch-and-wait strategy. Main-duct and mixed-type IPMNs are clear surgical diseases that require an oncological resection, while guideline recommendations for BD-IPMN are currently being discussed – especially with the regard to a defined size cutoff and other features of pre-malignancy. Therefore, individual decision-making is possible, and besides imaging features and potential symptoms, all other patient-related factors including age and comorbidities have to be weighed. In all types of IPMN, a lifelong surveillance or postoperative follow-up of the pancreatic remnant, respectively, is essential.

Cross-References

- ▶ Development of Novel Diagnostic Pancreatic Tumor Biomarkers
- ▶ Diagnostic Biomarkers
- ▶ Epidemiology and Prospects for Prevention of Pancreatic Cancer
- ▶ Evolution of Pancreatic Cancer Surgery
- ▶ Laparoscopic Surgery for Pancreatic Neoplasms
- ▶ The Molecular Pathology of Precursor Lesions of Pancreatic Cancer
- ▶ MRI and MRCP for Diagnosis and Staging of Pancreatic Cancer
- ▶ Surgical Resection for Pancreatic Cancer using the International Study Group of Pancreatic Surgery (ISGPS) Classifications
- ▶ Venous Resection in Pancreatic Cancer Surgery

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Laparoscopic Surgery for Pancreatic Neoplasms

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Contents

Abstract

After 20 years since its introduction, the laparoscopic approach has shown to be safe and reproducible in the surgical treatment of lesions in the pancreas, with the added benefits of reduced intraoperative blood loss and a shorter hospital stay. These benefits have been equally reproduced with surgical treatment of patients with pancreatic neoplasms. In the case of localized lesions in the body or tail of the pancreas, laparoscopic surgical treatment has proved equally effective as conventional open surgery in the short term, obtaining equivalent results from the oncological point of view. As for the laparoscopic surgical treatment of pancreatic head injuries, there is still a lack of available scientific evidence, but reported data show similar results to conventional surgery. Anyway, more studies

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are still needed to demonstrate the real role of the laparoscopic approach in the surgical treatment of patients with malignancies of the pancreas.

Keywords
Laparoscopic surgery · Pancreatic surgery · Pancreatic neoplasm · Pancreatic ductal adenocarcinoma · Pancreatic neuroendocrine tumor · Distal pancreatectomy · Pancreatoduodenectomy

Introduction

The first laparoscopic pancreatic resections were performed in 1994, when Gagner et al. [1] reported a total laparoscopic pancreaticoduodenectomy (LPD), and Cushieri et al. [2] published the first cases of laparoscopic distal pancreatectomy (LDP). Since then the interest on this type of resections grew exponentially, mainly for benign lesions due to uncertainty regarding oncologic outcomes. With the development and refinement of laparoscopic pancreatic surgery, parenchyma-sparing techniques such laparoscopic enucleation and central pancreatectomy extended the indications for the treatment of benign or borderline malignant pancreatic lesions.

In this chapter the current laparoscopic surgical techniques for the treatment of benign and malignant tumors of the pancreas will be discussed.

Laparoscopic Surgery for Exocrine Pancreatic Tumors

Twenty years after the introduction of laparoscopic surgery for exocrine pancreatic tumors, LDP has become a reality and is regarded by many as the standard of care for the treatment of left-sided pancreatic lesions. A recent meta-analysis from Mehrabi et al. [3] demonstrated the superiority of LDP over open distal pancreatectomy (ODP) in terms of blood loss, earlier oral intake, and length of hospital stay, without significant differences in terms of pancreatic fistula (PF) (21.8% vs. 21.6%) postoperative morbidity (34% vs. 38%), and mortality (0.4% vs. 1.1%). However, there is still a lack of randomized controlled trials between LDP and ODP, and it is very likely that such a trial will never be conducted.

Another important issue is the preservation of the spleen in LDP. The spleen does play an important immunological role; however preservation of the spleen at the time of LDP is controversial. Splenectomy has been reportedly associated with an increased postoperative morbidity, hematologic complications, and impaired primary immune response, as well as long-term increased risk for the development of certain malignancies [4, 5]. Therefore, spleen should be preserved whenever possible. Spleen-preserving DP (SPDP) is considered nowadays the procedure of choice for patients with benign or borderline malignant tumors of the pancreatic body and tail, as the majority of pancreatic neuroendocrine tumors (pNETs), although some authors have pointed out that it might be associated with an increased morbidity [6].

This procedure can be performed with or without splenic vessels preservation; the latter technique was described by Warshaw [7] where the splenic vascularization is fully dependent on the short gastric vessels. Adam et al. [8] published the combined experience of SPDP from Bordeaux and Barcelona comparing SPDP with and without splenic vessel preservation, and they observed equivalent results in operative time, blood loss, and conversion rate, but in the Warshaw technique (WT), they found a significant increase of splenic complications and a lower spleen preserving rate. Beane et al. [4] also found a significant advantage in clinically relevant PF, splenic infarction, overall morbidity, need for postoperative drainage placement, and shorter hospital stay in SPDP with vessel preservation. In addition, Fernandez-Cruz et al. [6] assumed that splenomegaly is a contraindication for VL-SPLDP due to insufficient nourishment of an increased mass by the short gastric vessels. On the other hand, SPDP seems to be associated with an increased risk for developing splenic vein thrombosis and subsequent left-sided portal hypertension, although the risk of variceal bleeding is unclear [9, 10].

Recently Sánchez Cabús et al. [11] published results after 115 consecutive LDP. SPDP was performed in 55.7% of the patients, with major postoperative complications in 25% of the patients and a clinically relevant PF rate of 11.3%, with a median postoperative hospital stay of 11 days. An additional analysis of that series of patients revealed that spleen preservation was associated with less major postoperative complications, independently of the surgical technique used, and authors identified splenectomy as an independent risk factor of postoperative major complications ($p = 0.019$, HR (95% CI): 4.617 (1.292–16.497)) [12]. Goh et al. [13] recently have published a comparative study of LPD (31 patients) versus robotic distal pancreatectomy (RDP, eight patients), finding equivalent outcomes but with an added advantage of RDP over LDP in terms of spleen preservation (3 (37.5%) vs. 25 (80.6%), $P = 0.016$) and splenic vessel preservation (5 (62.5%) vs. 4 (12.9%), $P = 0.003$), although associated with a longer median operation time (452.5 (range, 300–685) vs. 245 min (range, 85–430), $P = 0.001$).

The technical complexity of an LDP has been evaluated in terms of the learning curve, which is believed to be around ten cases, according to Braga et al. [14] and Ricci et al.'s [15] reports, although there are other factors that may influence this learning curve, such as the previous experience of the surgeons in both laparoscopy and pancreatic surgery. The issue of the closure of the pancreatic stump in LDP has been a matter of controversy. Pancreatic transection and stapler closure of the pancreas are widely adopted as the method of choice in LDP. Braga et al. [16] compared 100 LDP with 100 ODP, with a PF rate between the groups similar: 53% in the LDP group versus 51% in the ODP group, with 70% of PF in both groups being grade A, with stapler being used over 85% of the patients.

Whether these results might be applicable to patients with pancreatic ductal adenocarcinoma (PDAC) is a matter of controversy. In 2003, Strasberg described the radical antegrade modular pancreatosplenectomy (RAMPS), a new surgical technique for treating left-sided pancreatic cancer, which later has been adopted by the majority of groups and considered to be the gold standard procedure [17]. It aims to achieve a complete oncologic resection by keeping dissection into anatomical

planes and thus providing a radical operation. In addition, left-sided cancer is frequently associated to other organ infiltration, such as transverse colon or mesocolon or stomach. The RAMPS technique aims to increase the R0 resection rate and maximize the lymph node resection of the surgical specimen. There have been recently reports with few patients on the results of the laparoscopic RAMPS procedure for PDAC: Fernández-Cruz et al. [6] reported their results after laparoscopic RAMPS on ten patients obtaining free surgical margins in 90% of the patients and a median survival of 14 months. Song et al. [18] performed laparoscopic RAMPS in 24 patients with PDAC, with 22 out of 24 patients reaching R0 resection, a mean number of harvested lymph nodes of 10.3 ± 8.6, with a 2-year overall survival of 85.2%. Abu-Hilal et al. [19] have recently reported a R0 rate of 76% with a median node sample of 15 nodes and 1-year survival rate of 88%. Finally, a multicenter cohort study from four centers was published in 2015 by Sahakyan et al. [20] showing results after laparoscopic resection on 196 patients with PDAC, revealed a conversion rate of 2.6%, a clinically relevant postoperative pancreatic fistula (POPF) rate of 15.7%, with a median survival of 31.3 months, and an overall 5-year survival rate of 30%.

In 2010 Kooby et al. [21] published a retrospective study with data from nine academical centers in the United States comparing results from OPD with LDP. They were not able to find any differences neither in the positive margin rate or node retrieval nor in overall survival. They concluded that long-term survival was not influenced by the surgical approach. These findings were later confirmed by Magge et al. [22] in a comparative study with 62 patients with PDAC finding no differences in overall survival between both groups. Recently Sharpe et al. [23] published their study comparing 145 LDP with 625 ODP for PDAC again with no differences in terms of lymph node count, 30-day unplanned readmission, and 30-day mortality, with the added benefit of shorter hospital stay in LDP patients (6.8 ± 4.6 vs. 8.9 ± 7.5 days, $P < .001$). Finally, in 2015, Shin et al. [24] conducted a propensity score-matched analysis of PDAC patients resected by the ODP versus LDP with 51 patients in every group. Their results showed equivalent results in terms of primary outcomes of operative time, number of harvested lymph nodes, resection margin status, and secondary outcomes of frequency of POPF and complications, with no differences in patient survival. Stauffer et al. [25] reported in 2016 a comparative study of LDP versus ODP for PDAC, finding no differences in operative time, conversion to open surgery, POPF rate, and major postoperative complications. However, LDP was associated with a shorter hospital stay (5.1 vs. 9.4 days, $p = 0.0001$) and time to initiate adjuvant therapy (69.4 vs. 95.6 days, $p = 0.0441$). In addition, LDP was associated with more resected lymph nodes than ODP (25.9 vs. 12.7, $p = 0.0001$). Interestingly, survival rates at 1, 3, and 5 years were similar between LDP and ODP (69% vs. 78%, 41% vs. 44%, and 41% vs. 32%, respectively). Riviere et al. [26] published in 2016 a systematic review of data from 12 studies including 1,576 patients, 394 undergoing LDP, and 1,182 ODP for PDAC. None of the studies were randomized controlled trials, with all the evidence coming from retrospective cohort-like studies or case-control studies. Both techniques had equivalent outcomes in (LDP vs. ODP): short-term mortality (0.5% vs.

1%; odds ratio (OR) 0.48), serious adverse events (8.8% vs. 5.1%; OR 1.79), and clinically significant POPF (7.7% vs. 6.6%). Mean length of hospital stay was shorter by 2.43 days in the laparoscopic group than in the open group (MD −2.43 days). The results from all these studies are favorable to LDP and should ideally be confirmed in a randomized controlled trial. Tumors larger than 5–6 cm can be safely resected laparoscopically by the use of determinate surgical techniques that allow for a complete resection, as shown in a recent study by Fernández-Cruz et al. [27] showing results after LDP in 18 patients having tumors with a median size of 7 cm. R1 resections for exocrine pancreatic malignancies were found in 50% of patients. Morbidity (grade > II) was found in 16.6% of patients and 30-day mortality in one patient, with an overall median survival of 50 months and 29 months for patients with exocrine pancreatic malignancies.

Despite all these promising results, recently, De Rooij et al. [28] published a Dutch nationwide comparison of open versus LDP for both benign and malignant disease showing that LDP was associated with fewer major complications (16% vs. 29%; p: 0.02) and a shorter median hospital stay, but it only accounted for a 10% of all DP, so LDP is not universally accepted.

LPD is more technically demanding than LDP and is currently performed in few centers in the world. Liao et al. [29] published in 2016 a systematic review of minimally invasive PD (MIPD) reporting a conversion of 9.1%, average operative time of 422.6 min and average blood loss of 321.1 mL. The mean harvested lymph nodes were 17.1, and the rate of microscopically positive tumor margins was 8.4%. The cumulative morbidity was 35.9%, and a POPF was reported in 17.0% of cases. The average length of hospital stay was 12.4 days, and the mortality rate was 2.2%. Doula et al. [30] performed a systematic review of comparative studies between OPD with minimally invasive PD (LPD and robotic PD) including 14 articles. The conversion rate in LPD was between 0% and 15%, but the authors did not find any significant differences in resection margins, rates of POPF formation, bile leak, and delayed gastric emptying, reoperation rates, and intraoperative and postoperative mortality. The learning curve for LPD was studied by Wang et al. [31], which performed a CUSUM analysis and divided the learning curve into three separate phases: phase I was the initial learning period (cases 1–11), phase II represented the technical competence period (cases 12–38), and phase III was regarded as the challenging period (cases 39–57). They suggested that to attain a technical competence for performing LPD, a minimum of 40 cases should be required.

With respect to results after LPD for PDAC, there is less evidence than with LDP, and also there are no randomized controlled studies comparing open and laparoscopic approaches, only comparative and case-control studies. Adam et al. [32] conducted a study comparing OPD (6,078 patients) and minimally invasive PD (MIPD, 983 patients) with data from the National Cancer Database including years 2010 and 2011. The majority of hospitals (92%) performing MIPD were low volume (≤10 cases/2 years). The unadjusted 30-day mortality rate was 5.1% for MIPD versus 3.1% after open surgery. For patients with PDAC, there were no differences between MIPD and open PD after multivariable adjustment in number of lymph nodes removed, rate of positive surgical margins, length of stay, or readmissions.

However, 30-day mortality was higher for patients undergoing MIPD (OR 1.87 (95% CI 1.25–2.80), $p = 0.002$). De Rooij et al. [33] published in 2016 a systematic review and meta-analysis of comparative cohort and registry studies which included 1,833 patients. No differences were found in mortality or POPF. LPD was associated with prolonged operative, but lower intraoperative blood, less delayed gastric emptying, and shorter hospital stay. In addition, they found an increase in postoperative mortality in low-volume centers, which emphasizes the importance of high-volume centers on reaching good results. However, Dokmak et al. [34] published a comparative study between 46 patients undergoing LPD and 46 OPD. They found higher severe morbidity (28% vs. 20%, p: 0.32) in LPD due to grade C POPF (24% vs. 6%, p: 0.007), bleeding (24% vs. 7%, p: 0.02), and revision surgery (24% vs. 11%, p: 0.09), without any differences regarding the pathological reports between both approaches. Due to their results, these authors suggested that LPD should not be routinely performed for periampullary tumors. Experienced laparoscopic surgeons from the Mayo Clinic have shown the feasibility and safety of LPD compared to OPD for patients with PDAC [35–37], even in patients requiring major vascular resection and reconstruction. They found favorable results in LPD patients observing a significant reduction in blood loss (842 vs. 1,452 mL, $p < 0.001$), as in median hospital stay, (6 vs. 9 days, $p = 0.006$); no significant differences in the total number of complications (35% vs. 48%, $p = 0.24$) or severe complications ($\geq$ Clavien-Dindo III) (6.4% vs. 3.4%, $p = 0.51$) between the two groups. In their study, patients operated on for PDAC from LPD spent less time from surgery until the start of adjuvant chemotherapy than OPD patients (48 vs. 59 days, $p = 0.001$). These authors suggested that receiving adjuvant treatment in a timely and complete fashion should be an additional advantage of the laparoscopic approach.

One of the most important early steps in the performance of a PD consists of the correct assessment of SMA infiltration using the SMA first approach [38, 39], which is a technical modification of the standard PD. This has been a surgical step considered difficult by the laparoscopic approach. Recently, Pittau et al. have recently proved that the SMA first approach is feasible and safe to perform laparoscopically [40], helping to avoid futile resections.

In conclusion, the available evidence suggests that for patients having PDAC in the body/tail of the pancreas, LDP is a safe procedure and has shown benefits when compared to ODP in terms of blood loss and postoperative hospital stay without compromising long-term oncologic results. LPD for PDAC seems to be feasible, safe, and advantageous over OPD as well, but there is definitively less evidence in the literature.

Laparoscopic Surgery for Pancreatic Neuroendocrine Tumors

Pancreatic neuroendocrine tumors (pNETs) have been one of the most frequent indications for laparoscopic pancreatic surgery; the majority are benign or borderline malignant. In addition, some of these lesions are single and small that allow conservative pancreatic surgery, such as enucleation and central pancreatic resection.

In 2005 a European multicenter study on laparoscopic pancreatic surgery was published, which was one of the first reports to provide information about what was being performed at the time. The results showed that the majority of the cases reported (50/127 cases) were pNETs [41]. A recent study by de Rooij et al. [28] compared the Dutch experience between OPD and LDP showing that pNETs were the main indication for laparoscopic pancreatic resection (38%).

Indications for surgery for pNETs are well established [42–44], regardless that the resection is performed with conventional open or laparoscopic surgery. In functioning pNETs (F-pNETs), surgical resection is the treatment of election, and it is recommended for patients with single, sporadic tumors regardless of its size, since it eliminates or improves the symptomatology, even if the patient has distant metastases. However, even though the surgical approach should not modify the indications for resection, there are some F-pNETs that are better suited for laparoscopic resection than other tumors. For instance, insulinomas are tumors generally single and benignant without lymph node invasion. Therefore, due to its benign nature and if conditions are met, enucleation is a treatment of choice, avoiding the need for a more aggressive resection. On the other hand, other F-pNETs, such as gastrinoma, are not generally considered a good indication for laparoscopic surgery, since they can arise in the so-called gastrinoma triangle, and surgical exploration of duodenum is necessary in a large proportion of these patients [45].

In nonfunctioning pNETs (NF-pNETs), surgical indication is somewhat more controversial than for F-pNETs. Surgery is generally not indicated in small NF-pNETs (less than 2 cm), because they are asymptomatic, almost always discovered incidentally, and their probability of malignancy is believed to be very low. Edil et al. [46] showed that tumor size was associated with lymph node metastasis: <1 cm: 14%; 1–1.9 cm: 9%; 2–2.9 cm: 37%; 3–3.9 cm: 56%; 4–4.9 cm: 72%; and ≥ 5 cm: 56%. Thus, the accepted threshold for indicating resection is 2 cm, which is a size that associates greater proportion of nodal involvement. Another point of controversy is the surgical procedure needed for performing the resection, which depends on the size, main pancreatic duct involvement, location within the pancreatic gland, and, if available, results of preoperative biopsy. In general, parenchyma-preserving resection is indicated in small lesions, while more aggressive pancreatectomies should be recommended in larger lesions or when there is suspicion of a malignant tumor. Long-term results of laparoscopic pNETs resection were evaluated by Haugvik et al. [35], with an overall 5-year disease-specific survival rate of 90%, finding R2 resections, Ki67 $\geq$ 5%, and T4 tumors as bad prognosis risk factors.

Fernández-Cruz et al. [47] published their results after laparoscopic resection of pNETs. Laparoscopic enucleation resulted in the less blood loss compared with other techniques, but with a significantly higher postoperative morbidity due to a higher PF rate. Spleen-preserving DP also had higher complication rates than the conventional LDP due to spleen-related complications. However, results were excellent in terms of R0 resection, even in malignant tumors. Authors concluded that the benefits of minimally invasive surgery were manifested in the short hospital stay and acceptable pancreas-related complications in high-risk patients, with a high negative margin rate in patients with malignant tumors. Fernández-Cruz et al. [48] published

their results after laparoscopic enucleation for NF-pNETs in 30 patients. Clinically relevant PF, which is a main issue in this type of resection, occurred in 6.6% of the patients, and after a median follow-up of 48 months, only one patient developed lymph node and liver metastases.

In 2014, Drymousis et al. [49] published a systematic review on laparoscopic versus open surgery for pNETs. They included 906 patients, 22% of them resected laparoscopically, finding the known advantages of less intraoperative blood loss, less morbidity, and a shorter postoperative hospital stay. They were not able to find any differences on the PF rate, operative time or mortality between groups, even though the majority of the patients were resected by means of laparoscopic DP or enucleation.

In conclusion, laparoscopic pancreatic surgery has proven to be a feasible and safe approach and should be considered the standard of care in the management of patients with pNETs.

Conclusion

According to the available scientific evidence, laparoscopic surgery has been shown to be safe and effective for the treatment of neoplastic lesions of the pancreas, with the added short-term benefit of less intraoperative blood loss and a shorter hospital stay, obtaining similar long-term oncological results to those after conventional surgery, both for pancreatic ductal adenocarcinoma and pancreatic neuroendocrine tumors. However, the existence of stronger evidence from randomized controlled trials that definitely elucidate the role of laparoscopic surgery for the treatment of pancreatic neoplasms is still necessary.

Cross-References

- ▶ Borderline Resectable Pancreatic Cancer
- ▶ Miscellaneous Nonpancreatic Nonendocrine Tumors
- ▶ Sporadic Pancreatic Endocrine Tumors
- ▶ Surgical Resection for Pancreatic Cancer using the International Study Group of Pancreatic Surgery (ISGPS) Classifications

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Modern Japanese Approach to Pancreatic Cancer

Takao Ohtsuka and Masao Tanaka

Contents

Abstract

Principal clues to early diagnosis of pancreatic cancer including pancreatic duct dilation, diabetes, and intraductal papillary mucinous neoplasms are discussed, referring to Japanese contributions. The Japan Pancreas Society (JPS) has revised fourth edition of Clinical Guidelines for Management of Pancreatic Cancer, providing 51 clinical questions with graded evidence-based recommendations in 2016, and diagnosis, chemotherapy, radiation therapy, surgical resection, adjuvant treatments,

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and supportive therapy are addressed. “Borderline resectable (BR)” pancreatic cancer is a new concept, and JPS makes two categories of BR pancreatic cancer such as involvement of only portal vein (BR-PV) and involvement of major artery (BR-A). Extended resection cannot be actively advocated in patients with pancreatic cancer in the present daily practice, because several prospective randomized trials did not confirm the survival benefit of extended resection compared to standard resection. The analysis using data of the JPS Pancreatic Cancer Registry indicates that the 5-year survival rate of patients after resection of pancreatic cancer has been significantly improved after the introduction of gemcitabine into Japan. More recently, a Japanese group has demonstrated that S-1 adjuvant chemotherapy is superior to gemcitabine. Recent great interest in Japan is to clarify the significance of neoadjuvant treatments in resectable and BR pancreatic cancer.

Keywords
Pancreatic cancer · Japan Pancreas Society · JPS · Pancreatic Cancer Registry · Early diagnosis · IPMN · Borderline resectable · Extended resection · Adjuvant therapy · Neoadjuvant therapy

Current Status of Pancreatic Cancer in Japan

The number of pancreatic cancer-related death in Japan has increased to 31,716 in 2014, indicating that this disease has become the fourth leading cause of cancer death in Japan [1]. According to database of nationwide Pancreatic Cancer Registry conducted by the Japan Pancreatic Society (JPS) since 1981, the 5-year survival rate of overall patients with invasive pancreatic cancer has gradually increased from 6.7% to 13.0% during the recent three decades, and that of the patients who underwent pancreatectomy for invasive cancer has also increased from 10.9% to 18.8% [2] (Fig. 1). Despite the recent advances in imaging modalities and surgical techniques and the development of new anticancer agents, most pancreatic cancer patients have been still diagnosed as having a far advanced lesion, resulting in the poor prognosis. These data indicate the necessity of the establishments of diagnostic strategy for early-stage pancreatic cancer and multidisciplinary treatment strategy including resection for advanced pancreatic cancer to be cured.

Clinical Guidelines for Management of Pancreatic Cancer

The JPS published the first edition of evidence-based guidelines for the management of pancreatic cancer in 2006 [3] and fourth revised version in 2016 [4]. The first edited guidelines provided 29 clinical questions, while in the fourth edition, a total of 51 clinical questions are presented, indicating that management options and unresolved issues in the daily practice of the pancreatic cancer have been increasing. Answers are given to each clinical question with recommendations appropriately, graded based on evidences reported in relevant world literature.

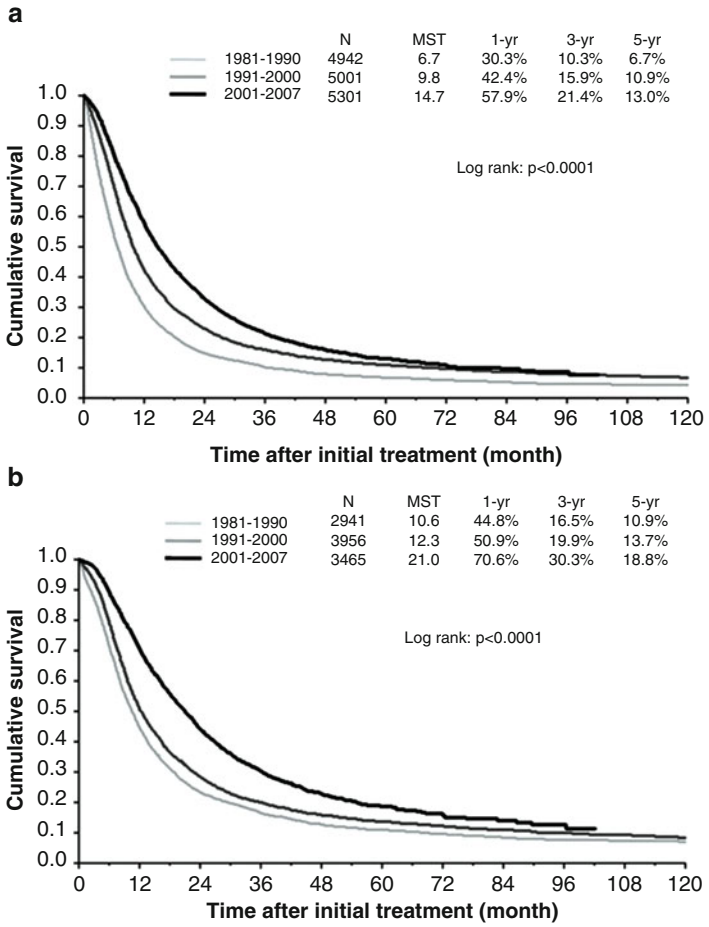

Fig. 1 Survival curves of overall patients with invasive pancreatic cancer (**a**) and those who underwent resection of invasive pancreatic cancer (**b**) in three periods. A table inset shows the number of patients (*N*), median survival time (*MST*) in months, and 1-year, 2-year, 3-year, and 5-year survival rates in each study period group (These figures are cited with permission from Ref. [2])

References with structured abstracts remade by the committee and contained in an attached CDROM are cited adequately. The entire guidelines cannot be translated here, but the clinical questions in the fourth edition are described in Table 1. Almost all aspects of the management of pancreatic cancer are addressed, including diagnosis, chemotherapy, radiation therapy, surgical treatments, adjuvant therapy, and supportive therapy. The guidelines also include some "perspectives" of the committee members, although this may be unusual for guidelines. Details of the "best supportive care" have been also described in the fourth edition of the guidelines. Algorithms for the diagnosis and treatment principals of pancreatic

Table 1 Fifty-one clinical questions in clinical guidelines for the management of pancreatic cancer 2016

1. Disease concept		
	I. Disease concept (DC)	
	DC-1	What are the risk factors to develop pancreatic cancer?
	DC-2	What is familial pancreatic cancer?
	DC-3	What is borderline resectable pancreatic cancer?
2. Diagnosis		
	II. Diagnosis (D)	
	D-1	How to detect pancreatic cancer?
	D-2	Diagnostic modality when pancreatic cancer is suspected
	D-2-1	Are CT and MRI useful to diagnose pancreatic cancer, when suspected?
	D-2-2	Is EUS useful to diagnose pancreatic cancer, when suspected?
	D-3	Next step to diagnose pancreatic cancer
	D-3-1	Is ERCP useful to diagnose pancreatic cancer?
	D-3-2	Is PET useful to diagnose pancreatic cancer?
	D-3-3	Is cytology/histology useful to diagnose pancreatic cancer?
	D-4	How to determine the stage of pancreatic cancer?
	D-5	How to determine the resectability of pancreatic cancer?
	D 6	Is it better to perform staging laparoscopy to determine the stage of pancreatic cancer?
	D-7	How to diagnose early stage pancreatic cancer possibly leading to long-term survival?
3. Treatment		
	A. Treatment of "resectable" pancreatic cancer (R)	
	III. Surgery (RS)	
	RS-1	Is resection recommended for resectable pancreatic cancer?
	RS-2	Is resection at high volume centers recommended for pancreatic cancer?
	RS-3	Does multidisciplinary treatment have a significant role in borderline resectable pancreatic cancer?
	RS-4	Does surgical resection have a significant role in pancreatic cancer with positive intraabdominal irrigation cytology?
	RS-5	Does preservation of the stomach have a significant role in pancreatoduodenectomy for pancreatic cancer?
	RS-6	Does combined resection of portal vein improve survival after resection of pancreatic cancer?
	RS-7	Does extended resection of retroperitoneal lymph nodes and neural plexus have a significant role in surgical treatment of pancreatic cancer?
	RS-8	Is prophylactic bypass recommended in pancreatic cancer proven to be unresectable during laparotomy?
	RS-9	Does laparoscopic pancreatectomy have a significant role in pancreatic cancer?
	RS-10	How to survey the patients undergoing resection of pancreatic cancer?
	RS-11	Is nutritional support recommended after resection of pancreatic cancer?

(*continued*)

Table 1 (continued)

IV. Adjuvant treatment (A)		
	RA-1	Is neoadjuvant therapy (chemoradiotherapy or chemotherapy) recommended for resectable pancreatic cancer?
	RA-2	Is intraoperative radiotherapy recommended for resectable pancreatic cancer?
	RA-3	Is postoperative adjuvant chemoradiotherapy recommended after resection of pancreatic cancer?
	RA-4	Is postoperative adjuvant chemotherapy recommended after resection of pancreatic cancer?
B. Treatment of locally advanced pancreatic cancer (LA)		
	LA-1	What is the primary treatment of unresectable locally advanced pancreatic cancer?
V. Radiation (LAR)		
	LAR-1	What kind of chemoradiotherapy is recommended for unresectable pancreatic cancer?
	LAR-2	How to determine the clinical target volume of external radiation therapy for unresectable pancreatic cancer?
	LAR-3	Does induction chemotherapy have a significant role in chemoradiotherapy for unresectable pancreatic cancer?
	LAR-4	Does intraoperative radiation therapy have an effect on unresectable pancreatic cancer?
	LAR-5	Does radiation therapy or chemoradiotherapy improve QOL of the patients with unresectable pancreatic cancer?
VI. Chemotherapy (LAC)		
	LAC-1	What is the primary agent of chemotherapy for unresectable locally advanced pancreatic cancer?
	LAC-2	(MC-2) Is secondary chemotherapy recommended for unresectable pancreatic cancer?
	LAC-3	(MC-3) What is the recommended period of chemotherapy for unresectable pancreatic cancer?
	LAC-4	(MC-4) Is immunotherapy recommended for unresectable pancreatic cancer?
C. Treatment of metastatic pancreatic cancer (M)		
VI. Chemotherapy (MC)		
	MC-1	What is the primary agent of chemotherapy for unresectable pancreatic cancer with distant metastasis?
	MC-2	(LAC-2) Is secondary chemotherapy recommended for unresectable pancreatic cancer?
	MC-3	(LAC-3) What is the recommended period of chemotherapy for unresectable pancreatic cancer?
	MC-4	(LAC-4) Is immunotherapy recommended for unresectable pancreatic cancer?
V. Radiation (MR)		
	MR-1	Is radiation therapy useful for bone metastasis from pancreatic cancer?
4. Supportive therapy		
VII. Stent insertion (ST)		
	ST-1	Is biliary drainage recommended for unresectable pancreatic cancer with obstructive jaundice?
	ST-2	Which is better as an approach of biliary drainage for unresectable pancreatic cancer with obstructive jaundice, percutaneous or endoscopic?

(*continued*)

Table 1 (continued)

	ST3-1	What kind of stent is recommended for preoperative biliary drainage in pancreatic cancer with obstructive jaundice?
	ST3-2	What kind of stent is recommended for biliary drainage in unresectbale pancreatic cancer with obstructive jaundice?
	ST-4	Which is recommended for gastric outlet obstruction in unresectable pancreatic cancer, gastrojejunostomy or stent?
VIII. Palliative medicine (PM)		
	PM-1	What is the effective care for psychological stress in pancreatic cancer patients and their family?
	PM-2	What is the effective treatment of upper abdominal pain and back pain in pancreatic cancer patients?
	PM-3	Is elemental diet support effective for the improvement of the condition of patients with unresectable pancreatic cancer?

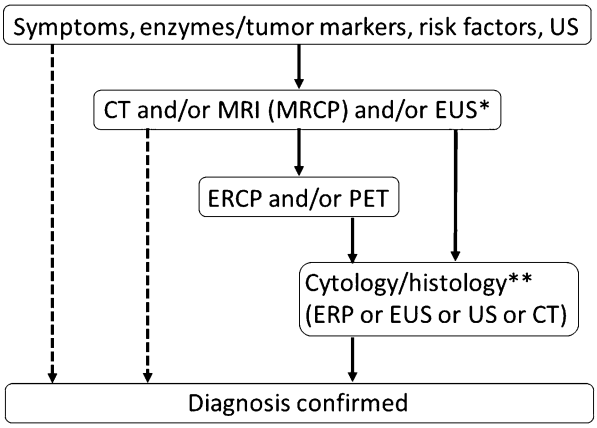

Fig. 2 Diagnostic algorithm (This figure is translated and cited with permission from Ref. [4]). *US* (percutaneous) ultrasonography, *CT* computed tomography, *MRI* magnetic resonance imaging, *MRCP* magnetic resonance cholangiopancreatography, *EUS* endoscopic ultrasonography, *ERCP* endoscopic retrograde cholangiopancreatography, *PET* positron emission tomography. *Enhanced CT and enhanced MRI + MRCP are preferable. EUS is possible at experienced institution. **Pathological diagnosis is preferable whenever possible

cancer presented in the guidelines are shown in Figs. 2 and 3. Briefly, any patients with symptoms suggestive of pancreatic cancer, elevation of serum and/or urinary amylase and/or tumor markers such as carbohydrate antigen 19-9 (CA19-9) and carcinoembryonic antigen (CEA), presence of multiple risk factors such as strong family history of pancreatic cancer, hereditary pancreatic cancer syndrome, diabetes mellitus, chronic pancreatitis, hereditary chronic pancreatitis, and cigarette smoking, and/or percutaneous ultrasonographic findings suggestive of pancreatic cancer should undergo enhanced computed tomography (CT) and/or magnetic

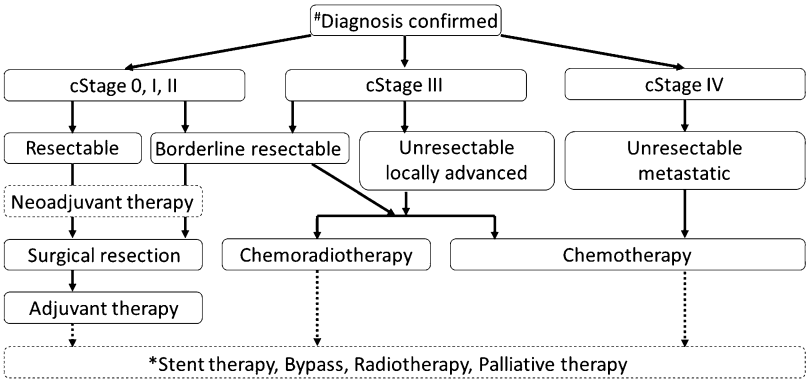

Fig. 3 Therapeutic algorithm (This figure is translated and cited with permission from Ref. [4]). Clinical stage (cStage) is determined according to the General Rules of Surgical and Pathologic Studies on Cancer of the Pancreas (seventh edition) (Ref. [5]). #Supportive treatment for pain, malabsorption, diabetes, anxiety is usually needed from the time of initial diagnosis. *Sometimes indicated in selected patients

resonance imaging/cholangiopancreatography (MRI/MRCP) and/or endoscopic ultrasonography (EUS). If these imaging studies have not confirmed the diagnosis, then the patients should be subjected to ERCP and/or positron emission tomography (PET). Pathological assessment (cytology/histology) under ERCP, EUS, or CT should be performed as much as possible (Fig. 2).

Seventh edition of General Rules of Surgical and Pathologic Studies on Cancer of the Pancreas [5] published by the JPS in 2016 has changed the classification of the location of pancreatic cancer (Fig. 4) and TNM classification (Fig. 5) to ensure the integrity with the Union for International Cancer Control (UICC) classification [6], and fourth edition of JPS guidelines for the management of pancreatic cancer [4] follows these revised classifications. The borderline between the pancreatic body and tail is set at left outer edge of aorta in the current rules, although this was previously set at the midline between the left outer edge of superior mesenteric vein/portal vein (SMV/PV) and left outer edge of pancreatic parenchyma. The category of T factor is almost the same with that of UICC [6], while the Japanese category divides T1 factor into three subcategories according to the tumor size and N1 factor into two subcategories according to the number of the metastatic lymph nodes, based on the assessment of the survival data between 2001 and 2007 in JPS Pancreatic Cancer Registry (Fig. 5) [5]. The JPS classification of peripancreatic lymph nodes is also demonstrated in Fig. 6. Nodal stations are classified into three groups according to the location of the lesion, either the head or body and tail of the pancreas or both (entire pancreas) (Fig. 7). Regional lymph node station includes Group 1 and 2. Stage IVA of locally advanced pancreatic cancer which was previously great concern of Japanese physicians in terms of the treatment strategy is currently compatible for stage IIA, IIB, or III in the seventh edition of JPS classification [5]. Of note, positive result of intraoperative irrigation cytology (CY1) does not yet belong to “M1” in the

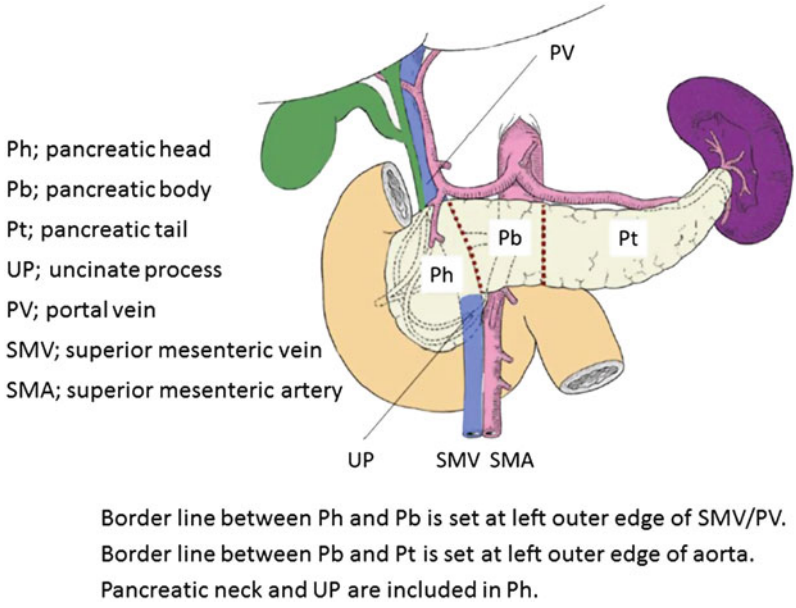

Fig. 4 Nomenclature of location of the tumor (This figure is translated and cited with permission from Ref. [5])

Primary tumor (T)

TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ
T1	Tumor limited to the pancreas, 2cm or less in greatest diameter
T1a	Tumor 5mm or less in greatest diameter
T1b	More than 5mm but 10mm or less in greatest diameter
T1c	More than 10mm but 20mm or less in greatest diameter
T2	Tumor limited to the pancreas, more than 2cm in greatest diameter
T3	Tumor extends beyond the pancreas but without involvement of the celiac axis or the superior mesenteric artery
T4	Tumor involves the celiac axis or the superior mesenteric artery

Distant metastasis (M)

M0	No distant metastasis
M1	Distant metastasis

Regional lymph nodes (N)

NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis
N1a	1 to 3 lymph nodes involvement
N1b	4 or more lymph nodes involvement

Stage

Stage 0	Tis	N0	M0
Stage IA	T1 (T1a, T1b, T1c)	N0	M0
Stage IB	T2	N0	M0
Stage IIA	T3	N0	M0
Stage IIB	T1 (T1a, T1b, T1c), T2, T3	N1 (N1a, N1b)	M0
Stage III	T4	Any N	M0
Stage IV	Any T	Any N	M1

Fig. 5 The stage of the disease in the Japan Pancreas Society (*JPS*) system according to the General Rules of Surgical and Pathologic Studies on Cancer of the Pancreas (seventh edition) [5]. This figure is translated and cited with permission from Ref. [5]. This classification ensures the integrity with the Union for International Cancer Control (*UICC*) classification [6]. The category of T factor is almost the same with that of UICC, while the JPS category divides T1 factor into three categories according to the tumor size, and N1 factor into two categories according to the number of the metastatic lymph nodes. Of note, positive result of intraoperative irrigation cytology (*CY1*) does not yet belong to "M1" in the seventh edited JPS classification

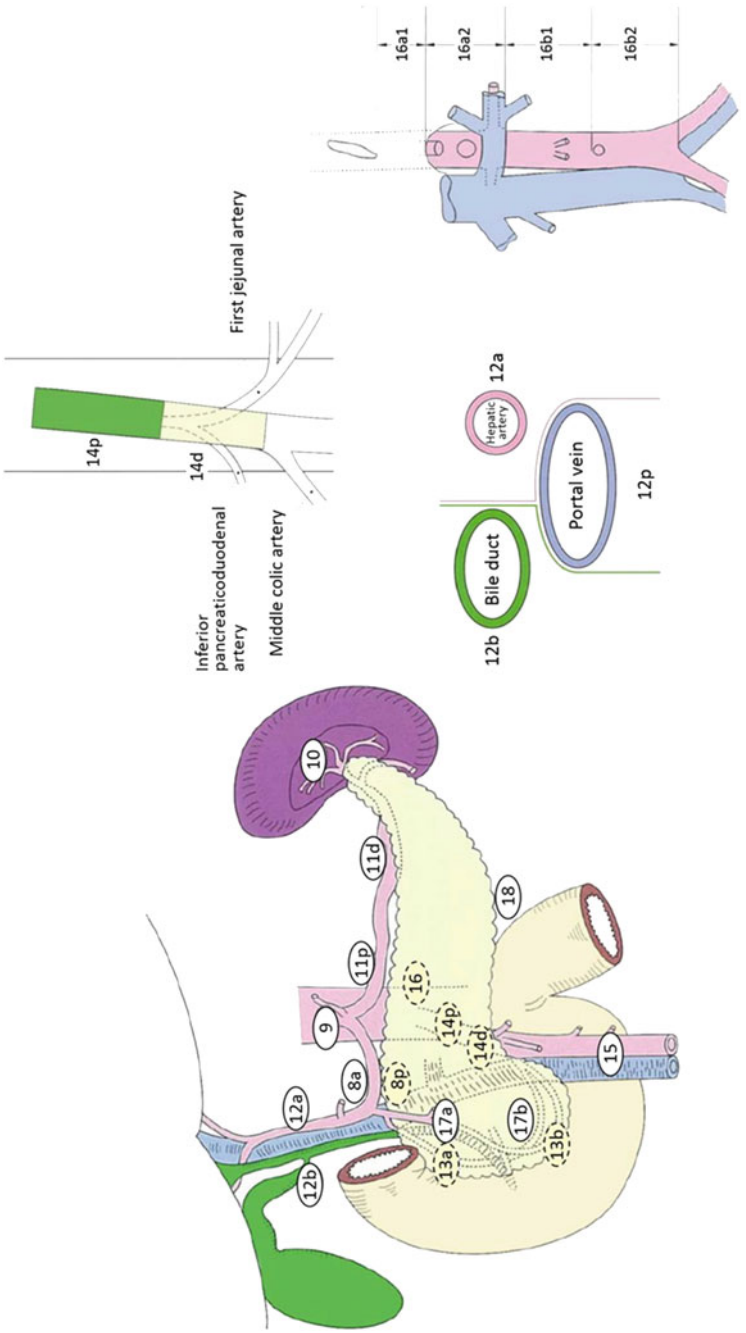

Fig. 6 Nomenclature of lymph node stations in the peri-pancreatic region (This figure is cited with permission from Ref. [5])

	Total pancreatectomy	Pancreato-duodenectomy	Distal pancreatectomy
Group 1	8a, 8p,10, 11p, 11d, 13a, 13b, 17a, 17b, 18	8a, 8p, 13a, 13b, 17a, 17b	10, 11p, 11d, 18
Group 2	5, 6, 7, 9, 12a, 12b, 12p, 14p, 14d	5, 6, 12a, 12b 12p, 14p, 14d	7, 8a, 8p, 9, 14p 14d
Group 3	1, 2, 3, 4, 15 16a2, 16b1	1, 2, 3, 4, 7, 9, 10, 11p, 11d, 15, 16a2, 16b1, 18	5, 6, 12a, 12b, 12p, 13a, 13b, 15, 17a, 17b, 16a2, 16b1

Fig. 7 Nomenclature of lymph node stations in the peri-pancreatic region and group numbers, according to the type of the operation (This figure is cited with permission from Ref. [5]). Regional lymph node station to determine the "N" factor includes Group 1 and 2

seventh edition of JPS TNM classification, because of the lack of evidences emphasizing the effects of CY1 on postoperative survival [7, 8].

Another new concept is "borderline resectable (BR)" pancreatic cancer. There are several definitions of BR-pancreatic cancer in Western countries [9, 10], while the JPS has made two categories of BR pancreatic cancer such as involvement of only PV/SMV (BR-PV/SMV) and involvement of major arteries (BR-A) irrespective of the presence or absence of PV/SMV involvement [4, 5], because Japanese surgeons aggressively perform pancreatectomy with combined resection of PV/SMV and usually determine the resectability based on the arterial involvement, not only on the PV/SMV involvement [11, 12]. BR-PV/SMV indicates the tumor involvement of PV/SMV less than 180° without invasion to or contact with superior mesenteric artery (SMA), celiac artery (CA), and common hepatic artery (CHA) and superior to the inferior margin of the duodenum, because reconstruction of SMV distal to the inferior margin of the duodenum is technically difficult [4, 5]. BR-A indicates tumor invasion to or contact with SMA or CA but without stenosis or deformity of SMA or CA or tumor invasion to or contact with CHA without invasion to or contact with proper hepatic artery and CA [4, 5]. If the tumor spread is matched with the criteria of both BR-PV/SMV and BR-A, then that will be managed as BR-A.

Clues to Early Diagnosis of Pancreatic Cancer

There have been a number of factors possibly leading to the diagnosis of pancreatic cancer. However, none have been successful to detect pancreatic cancer in early stages. Apart from well-known risk factors such as family history, chronic pancreatitis, cigarette smoking, etc., the following three factors (dilation of pancreatic duct, diabetes

mellitus, and intraductal papillary mucinous neoplasm of the pancreas) would deserve particular emphasis, because all of these are frequently encountered in clinical practice.

Dilatation of Pancreatic Duct

Dilation of the main pancreatic duct (MPD) can easily be demonstrated by ultrasonography and/or CT and may be the first sign to detect pancreatic cancer. However, specificity of this sign for the diagnosis of pancreatic cancer is rather low, since chronic pancreatitis also causes dilation of the MPD. As the most highly refined method to delineate the changes in the MPD, Inoue et al. [13] developed balloon catheter pancreatography and examined its diagnostic utility. The degree, length, and luminal deviation of a stenosis of the MPD as well as several branch duct findings such as paucity of branches around the MPD stenosis and irregular caliber changes were evaluated in 21 patients with pancreatic cancer and in 27 patients with chronic pancreatitis. Multivariate regression analysis demonstrated that only two findings were statistically significant in the differentiation of benign and malignant stenosis, i.e., severe stenosis and marked dilation of the upstream MPD [13]. These significant findings can now be evaluated by MRCP, and the role of ERCP is changing to sampling of the pancreatic juice for cytology and determination of molecular markers such as telomerase, hTERT, K-RAS, microRNA, etc. [14–21]. On the other hand, Iiboshi et al. [22] have recently demonstrated that endoscopic placement of naso-pancreatic drainage (ENPD) tube and subsequent pancreatic juice cytology in patients with MPD stricture increases sensitivity to detect pancreatic cancer. They examined 20 patients who had focal stenosis and distal dilation of the MPD by repeated pancreatic juice cytology via ENPD tube (average 5.3 time, range 2–11 times) and showed that the sensitivity, specificity, and accuracy of the cytological results of pancreatic juice were 100%, 83%, and 95%, respectively. Of note, among 15 patients with positive cytology via the ENPD tube subsequently diagnosed as having pancreatic cancer on resected specimen, seven had noninvasive carcinoma, all of whom had normal serum tumor marker levels and negative radiological mass finding. Kimura et al. [23] also reported their experiences of 24 patients with stage 0 and I pancreatic cancer according to JPS classification [5] and demonstrated that cytological examination during ERCP was 65% sensitive in preoperative diagnosis of such early-stage pancreatic cancer, whereas other imaging modalities were only 29–38% sensitive. Of note, 9 of 24 early-stage pancreatic cancers were diagnosed by ERCP/cytology alone. Despite the risk of acute pancreatitis, pancreatic juice cytology during ERCP still has important roles in the diagnosis of early-stage pancreatic cancer, which cannot be detected by any other imaging modalities.

Diabetes Mellitus

Diabetes mellitus is diagnosed in more than 50% of patients with pancreatic cancer and has long been considered to be one of factors to indicate the diagnosis of

pancreatic cancer. Ogawa et al. [24] prospectively studied 86 type 2 diabetic patients by ERCP and found six patients (7.1%) with pancreatic cancer. This very high prevalence was achieved by selection of patients using several criteria including (1) the onset of diabetes after the age of 55 without obesity, family history, or excessive alcohol ingestion, (2) acute exacerbation of preexistent diabetes, (3) loss of body weight despite good control of diabetes, (4) increased serum levels of amylase (>200 IU/L) and/or CA19–9 (>300 U/mL), and (5) ultrasonographic abnormalities of the pancreas. The study was continued until the total number of patients reached 197 and yielded the final prevalence of pancreatic cancer of 11.2% (data published only in Japanese). However, the most pancreatic cancers were diagnosed as advanced or unresectable condition, and thus, another screening system to detect early-stage pancreatic cancer in diabetic patients should be established. Chari et al. [25] reported that approximately 1% of diabetic subjects aged ≥50 years would be diagnosed with pancreatic cancer within 3 years of first meeting criteria for diabetes. The difference of these prevalence rates may be explained by more detailed criteria for patient selection.

Intraductal Papillary Mucinous Neoplasm (IPMN)

IPMN is a new clue to the diagnosis of pancreatic cancer [26]. Since Tanaka et al. first demonstrated case reports of pancreatic cancer concomitant with IPMN [27, 28], many other Japanese investigators have been much interested in this unique combination, and the reported incidence of concomitant pancreatic cancer ranges from 2.0% to 9.9% or 1.1% per year [29–32]. Kamata et al. [33] have recently conducted a prospective surveillance study of 102 IPMN patients and reported metachronous development of seven concomitant pancreatic cancers (7%). In this report, they showed the utility of EUS for the early detection of concomitant pancreatic cancers which were not diagnosed by CT and MRI/MRCP. On the other hand, Ohtsuka et al. [34] found 23 synchronous or metachronous pancreatic cancers occurring in 20 patients in a series of 179 patients who underwent resection of IPMNs. Seven of the 23 pancreatic cancers (30%) were of early stages (stage 0 to I according to JPS classification [5]), and sensitivities of CT, MRI/MRCP, and EUS to detect the stage 0 to I concomitant pancreatic cancers were 16%, 29%, and 29%, respectively, while sensitivity of ERCP/pancreatic juice cytology was 86%. Of note, three early-stage pancreatic cancers were diagnosed by ERCP/cytology alone, indicating that ERCP has an important role in the early diagnosis of distinct pancreatic cancers in patients with IPMNs. Ideno et al. found that IPMNs having concomitant pancreatic cancer are frequently of branch duct type, MUC2-negative gastric subtype, and of GNAS wild-type [35], and these molecular characteristics may lead to some insights to establish the diagnostic strategy for early detection of pancreatic cancer in patients with IPMNs. The JPS is now conducting a prospective multicenter surveillance study of branch duct IPMNs, and over 2,300 patients have been

registered between 2012 and 2014 (UMIN000007349). They will be surveyed for 5 years, using alternate CT and MRCP/EUS at every 6 months. Then, important informations with a high evidence level regarding the incidence of concomitant pancreatic cancer in patients with branch duct IPMNs and effects of alternate CT and MRCP/EUS on the early detection of concomitant pancreatic cancer will be obtained in 2019.

Strategy Against Locally Advanced Pancreatic Cancer

Extended Resection Versus Standard Resection

The treatment strategy for the Clinical Stage IVA pancreatic cancer in the previous sixth edition of JPS classification, indicating Clinical Stage IIA, IIB, and III in the current seventh edition, [5] remains controversial. This category includes resectable, borderline resectable, and unresectable locally advanced pancreatic cancers. Patients without invasion to major arteries (CHA in pancreatic head cancer, SMA in all pancreatic cancers) should undergo resection rather than chemoradiation. One prospective randomized study from Japan clearly showed a survival benefit of surgical resection compared to chemoradiation alone in patients with locally advanced pancreatic cancer invading to pancreatic capsule without involvement of the SMA or CHA and without distant metastasis [36, 37]. PV/SMV involvement does not preclude the indication for pancreatic resection as described above [11, 12], but the survival benefit of combined resection of the PV/SMV has not been proven yet. With regard to surgical treatments of pancreatic cancer, Japanese surgeons used to pursue cure of their patients with pancreatic cancer by means of extended resection with complete dissection of retroperitoneal lymph nodes and neural plexus in 1980s. One prospective randomized multi-institutional comparison conducted in Japan yielded no significant difference in overall 5-year survival rates between standard (No.13, 17 lymph node stations) (16%) and extended resection (No. 8, 9, 12, 13, 14, 16a2, 16b1, 17 lymph node stations, and circumferential nerve plexus of SMA) (6%) for pancreatic head adenocarcinoma [38]. Postoperative quality of life tended to be worse in the extended resection group as expected. In view of these results, the guidelines state [4] that extended resection cannot be actively advocated in patients with pancreatic cancer in the current daily practice. More recently, a Korean group has followed this Japanese investigation and demonstrated that extended resection (No. 5, 6, 8, 9, 12, 13, 14, 16a2, 16b1, 17 lymph node stations, and right-side nerve plexus of the SMA) does not provide a significant survival benefit compared with standard resection (No. 12 in part, 13, 17 lymph node stations) [39].

The survival benefit of extended resection in patients with lymph node metastasis may still justify the performance of D2 lymph node dissection (removal of Group 1 and 2 lymph nodes), because the precise status of lymph node metastasis can only be examined after surgery. In addition, the role of extended resection may

still have to be explored in earlier stage pancreatic cancer. Recent Japanese surgeons consider that standard resection includes pancreatectomy with lymph node dissection of Group 1 and 2 (D2), and therefore, the present JPS classification [5] defines the "regional" lymph nodes as Group 1 and 2 lymph nodes. Then, they limit the range of dissection area within Group 1 or extend the resection area to No.16 lymph node station, nerve plexus of the SMA, combined resection of PV/SMV, and sometimes CHA, according to the tumor spread as well as patients' general condition. Distal pancreatectomy with en bloc celiac axis resection is the representative extended pancreatectomy for pancreatic cancer designed by Japanese surgeons [40].

Adjuvant Treatment

Since the application of gemcitabine as a primary agent for the treatment of unresectable pancreatic cancer and as an adjuvant agent after resection of pancreatic cancer [41, 42], survival rates of overall patients as well as of those who underwent pancreatectomy have been gradually improving as described above (Fig. 1). More recently, Uesaka et al. [43] have conducted a randomized phase III trial of adjuvant chemotherapy comparing gemcitabine versus S-1 for patients with resected pancreatic cancer (JASPAC-01 study) and shown that hazard ratio for S-1 to gemcitabine was 0.56 (95% CI, 0.42–0.74, $p < 0.0001$ for non-inferiority, $p < 0.0001$ for superiority) based on the interim analysis. The 2-year survival rates were 53% (95% CI, 46–60) for gemcitabine and 70% (63–76) for S-1. These data indicate that S-1 adjuvant chemotherapy is superior to gemcitabine, and fourth edited JPS guidelines [4] recommend S-1 as the first choice of an adjuvant chemotherapeutic agent after resection of pancreatic cancer.

Because the effect of adjuvant therapy for pancreatic cancer is still limited, recent interest of Japanese investigators has been shifted to neoadjuvant therapy. Several Japanese prospective studies have shown the possible usefulness of neoadjuvant treatment for resectable or borderline resectable pancreatic cancer, using gemcitabine-based chemoradiation [44], gemcitabine plus S-1 [45], or carbon-ion radiotherapy [46], and lots of prospective randomized phase III trials are going on.

Conclusion

Japanese investigators have recently made great efforts to conduct multicenter or nationwide projects to provide high-quality evidences for the early diagnosis or the adequate treatment of pancreatic cancer, providing worldwide new insights as well as guidelines in accordance with Japanese situation. JPS plays important roles to arrange the clinical guidelines and the general rule of surgical and pathologic studies for the adequate management of Japanese patients with pancreatic cancer.

Key Practice Points

1. Pancreatic duct dilation, diabetes mellitus, and IPMNs may be clues to early diagnosis of pancreatic cancer.
2. Patients with locally advanced pancreatic cancer with no major arterial invasion should undergo resection rather than chemoradiation for better survival.
3. Definition of BR pancreatic cancer is different between Japan and Western countries, and the significance of this category during management of pancreatic cancer remains an unresolved issue.
4. Extended resection including para-aortic lymph nodes and neural plexus around the SMA has no overall survival benefit compared to standard resection.
5. The survival benefit of extended resection in patients with lymph node metastasis may still justify the performance of D2 lymph node dissection, because the precise status of lymph node metastasis can only be examined after dissection.
6. Adjuvant chemotherapy with S-1 or gemcitabine is recommended after resection of pancreatic cancer.

Future Research Directions

1. A breakthrough to more efficient methods for early detection of pancreatic cancer is urgently needed.
2. High-risk factors predisposing to pancreatic cancer must be recognized more widely to subject patients to imaging studies of the pancreas.
3. The role of extended resection may still have to be explored in earlier stages of pancreatic cancer.
4. Effects of neoadjuvant treatments to further improve the prognosis of resected pancreatic cancer should be investigated in prospective randomized trials.

Cross-References

- ▶ Adjuvant Chemotherapy in Pancreatic Cancer
- ▶ Borderline Resectable Pancreatic Cancer
- ▶ Clinical Decision-Making in Pancreatic Cancer
- ▶ Development of Novel Diagnostic Pancreatic Tumor Biomarkers
- ▶ Epidemiology and Prospects for Prevention of Pancreatic Cancer
- ▶ Management of Cystic Neoplasms of the Pancreas Including IPMNs
- ▶ Neoadjuvant Chemotherapy in Pancreatic Cancer
- ▶ New Japanese Classification of Pancreatic Cancer
- ▶ Surgical Resection for Pancreatic Cancer Using the International Study Group of Pancreatic Surgery (ISGPS) Classifications
- ▶ Venous Resection in Pancreatic Cancer Surgery

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Neoadjuvant Chemotherapy in Pancreatic Cancer

Theodoros Michelakos and Cristina R. Ferrone

Contents

Abstract

More than 30% of pancreatic ductal adenocarcinoma (PDAC) patients present with borderline resectable (BR) or locally advanced (LA) disease. Historically, this patient population had a poor prognosis, with the majority not being offered an operation. Following the promising results of modern combination regimens such as FOLFIRINOX (5-FU, oxaliplatin and irinotecan) and gemcitabine plus nab-paclitaxel for patients with metastatic PDAC, these regimens have been utilized in patients with BR or LA disease to render them resectable. Indeed, neoadjuvant FOLFIRINOX increases resectability of LA PDAC up to 44%, with

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margin-negative resection rates and overall survival rates comparable to upfront resectable patients. Neoadjuvant chemotherapy also aids in obviating adjuvant therapy, which is frequently not initiated or completed due to the morbidity associated with pancreatic operations. Based on the encouraging results in locally advanced and borderline patients, neoadjuvant chemotherapy may also be of use in patients presenting with resectable disease. Neoadjuvant therapy may aid in screening patients with aggressive disease who progress on neoadjuvant therapy, and therefore may not benefit from an operation. Clinical trials currently underway will provide further information on the efficacy of modern neoadjuvant therapies for PDAC patients.

Keywords
Pancreatic ductal adenocarcinoma · Neoadjuvant chemotherapy · FOLFIRINOX · Gemcitabine and nab-paclitaxel · Locally advanced pancreatic adenocarcinoma · Borderline resectable pancreatic adenocarcinoma

Introduction

The incidence of pancreatic ductal adenocarcinoma (PDAC) continues to increase worldwide. In the United States, it is currently the third leading cause of cancer death [1]. Approximately, 15%–20% of patients present with resectable disease, yet 80% of these patients already have cancer in their locoregional lymph nodes [2, 3]. More than 30% of PDAC patients present with borderline resectable (BR) or locally advanced (LA) disease [4]. Historically, these patients have a poor survival of only 8–12 months from the time of diagnosis [2, 5]. In the majority of cases, gemcitabine and/or 5-FU-based chemoradiation was utilized in an attempt to render patients resectable, but often the therapy was palliative with less than one-third of patients down-staged and resected [5–7]. Even after resection, survival remained poor with a median of 20 months (range 9–62 months) [5, 7].

In 2011, the PRODIGE 4/ACCORD 11 trial [8] demonstrated that the combination of 5-FU, oxaliplatin, and irinotecan (FOLFIRINOX) led to improved overall and progression-free survival compared to gemcitabine alone for patients with metastatic PDAC. Based on these results, FOLFIRINOX became the standard of care for patients with metastatic PDAC. With the promising outcomes in the metastatic setting, FOLFIRINOX has been utilized in patients with BR or LA disease. Many of these patients also received chemoradiation, in an attempt to render them resectable. While no results from randomized control trials evaluating the efficacy and safety of FOLFIRINOX in the neoadjuvant setting have been published, retrospective studies have demonstrated encouraging results [9–13].

Similarly, nab-paclitaxel in combination with gemcitabine in the MPACT trial [14] demonstrated an improved overall survival and progression-free survival when compared to gemcitabine alone for the treatment of metastatic PDAC. Subsequently, this combination therapy has also been utilized in the neoadjuvant setting for patients

with borderline or LA disease. Other gemcitabine-based neoadjuvant regimens, such as gemcitabine plus S1 [15] and gemcitabine plus oxaliplatin [16], have also been evaluated in clinical trials with promising results.

Definitions

Resectable/Borderline Resectable/Locally Advanced

Accurate staging and selection of patients is crucial to maximizing the benefits and minimizing the risks of treatment. PDAC can be classified in a spectrum from resectable to unresectable, based on the presence of distant metastases and the tumor's relationship to vascular structures and other organs. It is important that the classification and management of each patient is discussed in a multidisciplinary team. The PDAC multidisciplinary board at the Massachusetts General Hospital (MGH) consists of two or more pancreatic surgeons, one or more gastrointestinal radiologists, two or more medical oncologists, and one or more gastrointestinal radiation oncologist. To determine resectability, the Americas Hepato-Pancreato-Biliary Association (AHPBA)/Society of Surgical Oncology (SSO)/Society for Surgery of the Alimentary Tract (SSAT) consensus criteria [17] are used.

Rationale for the use of Neoadjuvant Therapy in PDAC

Resection offers the only chance for potential cure of patients with PDAC. Additionally, patients who have not received neoadjuvant therapy and are resected with a negative resection margin >1 mm survive significantly longer than patients with an R1 resection or unresectable LA disease [18] (Fig. 1). Therefore, it is extrapolated that neoadjuvant therapy which down-stages patients, so that a margin negative resection can be achieved, could be beneficial.

Similar to other cancers, effective neoadjuvant therapy does not only down-stage cancers and treat undetected early micro-metastases, but also aids in patient selection. While the operative outcomes of pancreatic resections continue to improve, there is still a relatively high morbidity (30–60%) and mortality (1–5%) at high volume centers [19–21]. Neoadjuvant therapy can aid in selecting the best candidates for surgical resection by offering those with good biology who respond to neoadjuvant therapy an operation, while avoiding a resection in patients with aggressive biology which progresses on neoadjuvant therapy. Neoadjuvant therapy may also obviate the need for additional adjuvant therapy. In large trials, such as ESPAC-3 and CONKO 1, adjuvant therapy was not initiated or completed in approximately 10% and 40% of patients, respectively, due to complications associated with the pancreatic operations, disease progression, patients' decision, or toxicity [22, 23].

Patients for whom neoadjuvant therapy is considered should first obtain a tissue diagnosis to confirm the diagnosis. Second, eligible patients should have an adequate

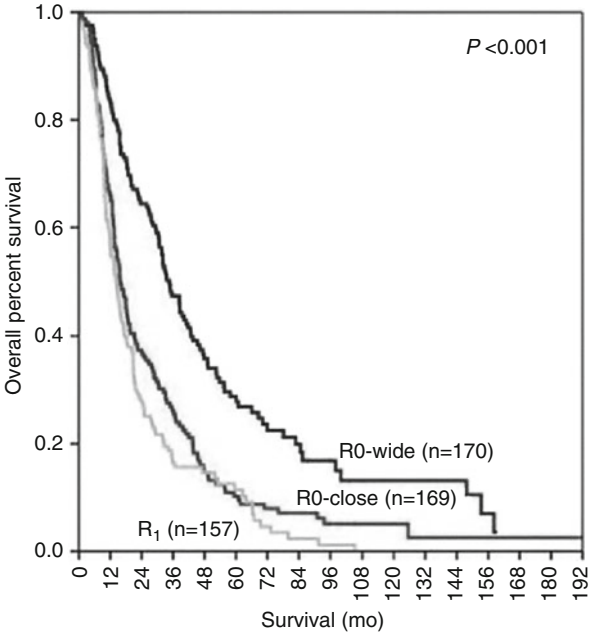

Fig. 1 Improved overall survival of PDAC patients with margins > 1 mm. Kaplan-Meier overall survival curve: R0 resection with wide margin >1 mm versus R0 resection with close (1 mm) margin versus R1. Median survival was 35 versus 16 versus 14 months, respectively. P < 0.001 (Konstantinidis et al. [18])

performance status to withstand neoadjuvant regiments (Eastern Cooperative Oncology Group [ECOG] 0 or 1 [24]). Third, patients should undergo biliary drainage if they present with obstructive jaundice.

Locally Advanced PDAC

No prospective randomized trials with modern chemotherapy have been completed in patients with LA PDAC. FOLFIRINOX is the most widely studied modern neoadjuvant combination regimen, yet the data are mainly derived from small-sample retrospective studies (Table 1). Even fewer data are available for gemcitabine plus nab-paclitaxel. Among patients with BR or LA PDAC who received neoadjuvant FOLFIRINOX alone, 0%–33% were down-staged and resected [25–28]. When FOLFIRINOX was followed by neoadjuvant chemoradiation, the frequency of resectability is increased up to 44% [9–12, 29–34].

Neoadjuvant FOLFIRINOX seems to increase the frequency of negative resection margins. In cohorts of resected FOLFIRINOX patients, an R0 margin was achieved in 41–100% of patients [3, 9–12, 28–32, 34–36]. Rombouts et al. in a systematic review of 14 studies calculated that the total R0 frequency was 77% [37]. The MGH experience in 110 patients was an R0 (>1 mm) resection rate of 81% (unpublished data). This compares favorably with resectable patients who went directly to the operating room and had an R0 rate of approximately 70–80% [22,

Table 1 List of studies evaluating the role of neoadjuvant chemotherapy in locally advanced, borderline resectable, and resectable pancreatic ductal adenocarcinoma

Author	Year	Study type	N	Neoadjuvant therapy	Resected, N (%)	R0, N (% of resected)	OS, months	DFS, months
Faris [9]	2013	Retrospective, single-center	22	FOLFIRINOX (n = 22) followed by chemoradiation (n = 20)	5 (23%)	5 (100%)	Median, from start of FOLFIRINOX: 24.7 (IQR: 19.0–30.3)	Median, from start of FOLFIRINOX: 11.8 (IQR 8.6–15.1)
Marthey [31]	2014	Prospective, multicenter	77	FOLFIRINOX (n = 77) followed by chemoradiation (n = 54)	28 (36%)	25 (89%)	Median, from start of FOLFIRINOX: 21.1 (IQR: 12.3–29.9)	Median, from start of FOLFIRINOX: 18.5 (IQR 12.9–24.1)
Pietrasz [35]	2015	Prospective, multicenter	33	FOLFIRINOX (n = 33) followed by chemoradiation (n = 22)	33 (100%) (inclusion criterion)	28 (85%)	For both LA and BR: OS: 59.2 (95% CI 45.7–72.7)	For both LA and BR: DFS: 17.2 (95%CI 11.3–23.0),
Sadot [12]	2015	Retrospective, single-center	101	FOLFIRINOX (n = 101) followed by chemoradiation (n = 63)	31 (31%)	16 (52%)	Median, from start of FOLFIRINOX: 26.0 (IQR: 19.3–32.7)	Median, from start of FOLFIRINOX: 16.0 (IQR 13.3–18.7)
Hackert [36]	2016	Retrospective, single-center	125	FOLFIRINOX (n = 125)	76 (61%)	31 (41%)	Median, from resection: 16.0	–
Borderline resectable								
Katz [51]	2008	Retrospective, single-center	160	Gemcitabine (alone or in combination) followed by/or chemoradiation	66 (41%)	62 (94%)	Median 18	–
Katz [47]	2016	Prospective, multi-center, single-arm trial	22	FOLFIRINOX followed by chemoradiation	15 (68%)	14 (93%)	Median, from registration: 21.7 (95% CI, 15.7-N/A)	Progression-free survival rate at 12 months: 59% (95% CI 42%–84%)

(*continued*)

Table 1 (continued)

Author	Year	Study type	N	Neoadjuvant therapy	Resected, N (%)	R0, N (% of resected)	OS, months	DFS, months
Kim [46]	2016	Retrospective, single-center	26	FOLFIRINOX alone (n = 22), or followed by chemoradiation (n = 4)	26 (100%) (inclusion criterion)	24 (92%)	Median OS: not reached (median follow-up: 27.6)	Median 22.6
Paniccia [49]	2014	Retrospective, single-center	18	FOLFIRINOX (n = 18) followed by chemoradiation (n = 8)	17 (94%)	17 (100%)	Median OS: not reached (median follow-up from start of FOLFIRINOX: 14.5)	Progression-free survival rate at 12 months after start of FOLFIRINOX: 73.1% (95%CI 43%–89%)
Pietrasz [35]	2015	Prospective, multicenter	47	FOLFIRINOX (n = 47) followed by chemoradiation (n = 30)	47 (100%) inclusion criterion	39 (83%)	For both LA and BR: OS: 59.2 (95% CI 45.7–72.7)	For both LA and BR: DFS: 17.2 (95%CI 11.3–23.0),
Resectable								
Heinrich [56]	2008	Prospective phase II trial, single-center	28	Gemcitabine plus cisplatin	25 (89%)	20 (80%)	Actuarial OS: 26.5 (95%CI 11.4–41.5)	Actuarial recurrence-free survival: 9.2 (95%CI 5.6–12.9)
Palmer [57]	2007	Randomized phase II trial, single-center	50	Gemcitabine alone (n = 24) or gemcitabine plus cisplatin (n = 26)	27 (54%)	18 (67%)	Median, from randomization; 13.6 (95%CI 9.1–24.2)	–
Varadhachary [58]	2008	Prospective phase II trial, single-center	90	Gemcitabine plus cisplatin followed by chemoradiation	52 (58%)	50 (96%)	Median 17.4 (95%CI 14.5–20.3)	Median progression-free survival 13.2 (95%CI 11.9–14.4)

OS overall survival, *DFS* disease-free survival, *IQR* inter-quartile range, *CI* confidence interval

23, 38] as well as those who underwent only neoadjuvant chemoradiation where an R0 of 11%–32% was achieved [39].

Survival

A recent patient-level meta-analysis including 13 studies with 355 patients with LA PDAC treated with FOLFIRINOX +/− chemoradiation [13] reported a pooled median overall survival calculated from the start of FOLFIRINOX of 24.2 months (range 10.0–32.7 months) and a progression-free survival of 15.0 months (range 3.0–20.4 months). In the MGH experience of 141 BR/LA PDAC patients, a median overall survival of 34.2 months (interquartile range: 19.9–55.5) from the time of diagnosis was observed. Survival was even better for resected patients who reached an overall survival of 37.7 months (interquartile range: 23.0–55.5) (Fig. 2) and a disease-free survival of 29.1 months (interquartile range: 15.6-not reached) (unpublished data). Although these results are not derived from clinical trials and might suffer from selection bias, they are clearly better than those from historic studies treating LA PDAC patients [5, 7].

Toxicity

The main drawback of combination therapies such as FOLFIRINOX or gemcitabine plus nab-paclitaxel is their high frequency of toxicities when compared to

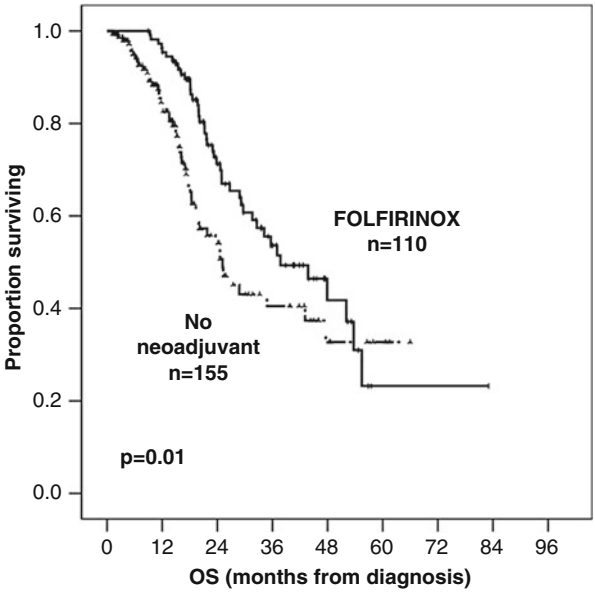

Fig. 2 BR/LA PDAC patients who received neoadjuvant FOLFIRINOX followed by resection had a better overall survival compared to resectable patients who underwent upfront resection. Kaplan-Meier overall survival curves: BR/LA who received neoadjuvant FOLFIRINOX followed by resection versus resectable patients who underwent upfront resection. Median overall survival from diagnosis was 37.7 (interquartile range 23.0–55.5) versus 25.1 months (interquartile range 15.4–not reached), respectively. P = 0.01

monotherapies. The PRODIGE 4/ACCORD 11 trial demonstrated increased toxicity for FOLFIRINOX when compared to gemcitabine for metastatic PDAC [8]. The most common side effects encountered with FOLFIRINOX are neutropenia, thrombocytopenia, diarrhea, vomiting, sensory neuropathy, and fatigue. Specifically, a systematic review of studies on neoadjuvant FOLFIRINOX therapy of LA PDAC [37] calculated a total frequency of grade 3–4 toxicity of 23% (51/220), while according to a recent meta-analysis [13], the pooled grade 3–4 adverse rate was 19.6% for neutropenia, 5.9% for thrombocytopenia, 8.2% for diarrhea, 8.8% for emesis, and 11.7% for fatigue. Interestingly, in the PRODIGE 4/ACCORD 11 trial, FOLFIRINOX reduced the quality of life impairment compared to gemcitabine, making patients actually feel better despite the toxicity associated with the chemotherapy [40].

Borderline Resectable PDAC

In an attempt to increase the rate of margin-negative resections and to improve outcomes, neoadjuvant chemotherapy has been suggested not only in LA PDAC patients, but also in borderline resectable patients. Although many studies have examined neoadjuvant therapy in this population, most are small single-center retrospective studies with mixed borderline/locally advanced cases [41–50].

In the largest study including only BR cases by Katz et al. [51], 125 of 160 BR PDAC patients completed neoadjuvant therapy (chemotherapy, chemoradiation, or both) and restaging. Of those, 79/125 (63%) underwent an operation after neoadjuvant therapy and 66/125 (42%) were resected. Negative margins were achieved in 94% of resected patients and overall survival for resected patients was 40 months.

In the era of modern neoadjuvant chemotherapy, only limited number of cases have been reported [44–50]. In a patient-level meta-analysis (presented as poster at the 2017 Pancreas Club by Suker et al.) including 17 studies with 250 borderline patients, the median OS was 18 months and the margin-negative resection rates ranged from 50% to 100% [52]. Furthermore, the initial results of the Alliance for Clinical Trials in Oncology Trial A021101 [47] demonstrated that of the 22 BR PDAC patients who received modified FOLFIRINOX followed by chemoradiation, 15 (68%) underwent an operation and 12 (80%) had negative margins (>1 mm). The median overall survival was 22 months from the registration to the trial.

Resectable PDAC

The best performers, resectable patients, have historically been the minority of patients who present to the physician. Unfortunately, even in this cohort of patients, 25% die within 12 months of their pancreatic resection (Fig. 3) [38]. Improved patient selection is desperately needed to avoid subjecting patients to a large and complex operation associated with significant morbidity and mortality [53]. Neoadjuvant chemotherapy is one approach to improve patient selection.

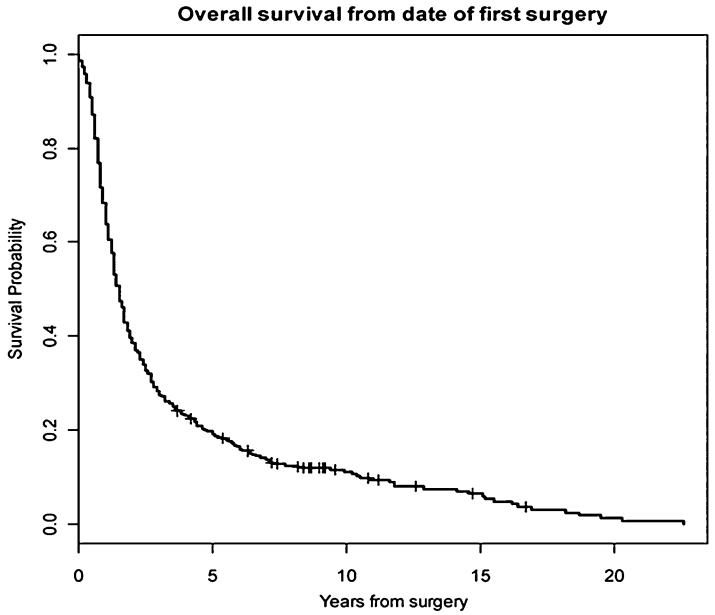

Fig. 3 Pancreatic ductal adenocarcinoma has poor prognosis. Kaplan-Meier overall survival curve: 499 patients who underwent an operation for their PDAC at MGH between 1985 and 2006. Actual survival rate at 5 and 10 years was 19% and 10%, respectively (Ferrone et al. [53])

At least three meta-analyses have investigated the role of neoadjuvant therapy in patients presenting with resectable disease [5, 7, 54]. The pooled frequency of resected patients ranged from 66% to 82% and pooled R0 rates ranged from 82% to 89%. In the meta-analysis by Gillen et al. [5], the pooled median survival of patients initially deemed resectable was 23.3 months for those who were resected and 8.4 months for those who were not. Among prospective studies [7], the weighted mean of the median survival was 18.8 months for all patients initially deemed resectable (resected or not resected), while in the meta-analysis of phase II trials [54], the median survival of this patient population was 23.0 months from diagnosis or the start of neoadjuvant therapy.

Utilizing data from the National Cancer Database in a propensity score-matched analysis [55], resectable Stage I/II patients receiving neoadjuvant therapy followed by an operation had a better survival compared to those who underwent upfront resection (26 vs. 21 months). Additionally, patients in the neoadjuvant group had a lower T stage, lower frequency of lymph node positivity (48% vs. 73%), and a higher negative margin resection rate (83% vs. 76%).

Neoadjuvant Therapy for Resectable PDAC in the era of FOLFIRINOX

As demonstrated above, patients who initially presented with BR or LA advanced disease received neoadjuvant FOLFIRINOX prior to being resected, than patients

with initially resectable disease who received no neoadjuvant therapy [3]. Indeed, the pooled median overall survival of 24.2 months reported in the LA PDAC patient-level meta-analysis by Suker et al. [13] is comparable to the 23.6 months documented in resectable PDAC patients receiving adjuvant gemcitabine in the ESPAC-3 trial [23]. Based on these findings, it has been advocated that neoadjuvant FOLFIRINOX could be used routinely in patients with resectable PDAC [56–58]. Currently, several clinical trials (NCT02782182, NCT02178709, NCT02172976, NCT01560949, NCT02959879, NCT02047474, NCT01660711, NCT02345460) are assessing the benefit of neoadjuvant FOLFIRINOX in the setting of resectable PDAC. Furthermore, for initially resectable patients, NCT02243007 performed at MGH and the SWOG S1505 trial (NCT02562716) are comparing perioperative FOLFIRINOX versus gemcitabine plus nab-paclitaxel. The ESPAC-5F trial (ISRCTN89500674) is comparing resection followed by adjuvant 5-FU versus neoadjuvant gemcitabine plus capecitabine followed by operation versus neoadjuvant FOLFIRINOX followed by resection versus chemoradiation followed by resection.

Response and Follow-up

If patients treated neoadjuvantly progress during treatment, the treatment regimen should be altered. Currently, there is no consensus regarding adjuvant therapy in patients receiving neoadjuvant therapy. MGH follows the NCCN guidelines and checks CA 19–9 every 3 months and imaging every 6 months.

Predictors of Response and Resectability

Several studies [3, 59, 60] have demonstrated that radiological imaging after the completion of neoadjuvant therapy is not a reliable predictor of resectability. Specifically, in the MGH experience including 40 borderline and LA PDAC patients treated neoadjuvantly with FOLFIRINOX +/− chemoradiation, 30% were classified as resectable preoperatively, but 92% of patients underwent an R0 resection. Along the same lines, Katz et al. demonstrated that among patients with borderline PDAC treated neoadjuvantly with gemcitabine-based therapy +/− CRT or CRT alone, only 0.8% of tumors were deemed radiologically resectable preoperatively, but 66% underwent a resection. The inability of radiologic imaging to determine resectability could be attributed to the significant fibrosis which replaces viable tumor tissue in response to neoadjuvant therapy [61, 62]. Currently, imaging is not able to differentiate between viable tumor and fibrosis (Fig. 4).

Several studies have attempted to identify predictors of response to neoadjuvant therapy. Hohla et al. [27] suggested that female gender might be a predictor of

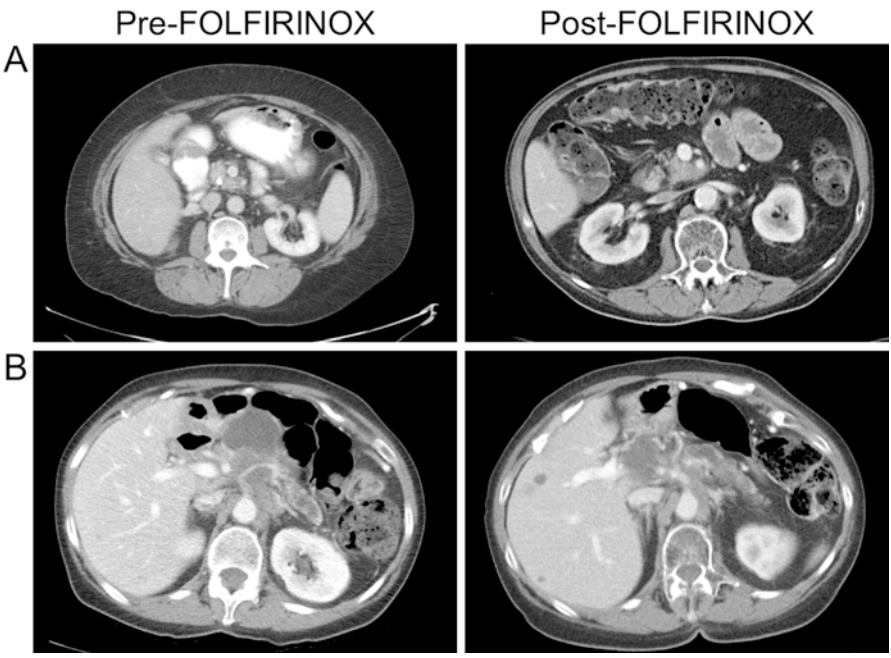

Fig. 4 Imaging does not predict resectability after neoadjuvant FOLFIRINOX. (**a**) 41-year-old female presenting with a 3.6 cm pancreatic mass involving the superior mesenteric artery. CA 19–9 at presentation was 985 U/mL. After 4 months of FOLFIRINOX and 50.4Gy of chemoradiation, her CA 19–9 was 37 U/mL. Final pathology revealed a 1.6 cm T2N0M0 PDAC with negative margins. (**b**) 69-year-old female presenting with a 4.1 cm pancreatic mass involving of the celiac trunk. CA19–9 at presentation was 13,735 U/mL. After 4 months of FOLFIRINOX and 50.4Gy of chemoradiation, her CA 19–9 was 25 U/mL. The CT scan demonstrated a 1.9 cm pancreatic lesion. Final pathology revealed a 2 cm T2N0M0 PDAC with negative margins

response since females had a significantly higher disease control rate of 91.7% compared to 48.0% in male patients ($p = 0.001$). More recently, Bednar et al. [63] demonstrated that pancreatic head/neck lesions (OR 0.307, $P = 0.033$) and SMA involvement (OR 0.285, $P = 0.023$) were independent predictors of resection in LA PDAC patients treated neoadjuvantly with FOLFIRINOX or nab-paclitaxel plus gemcitabine. CA 19–9 levels have also been suggested as a marker of resectability: Boone et al. [64] demonstrated that in borderline resectable patients treated with neoadjuvant therapy (Gemcitabine-based or FOLFIRINOX), a CA 19–9 response of >50% predicted an R0 resection (odds ratio 4.2; $p = 0.05$), while in borderline resectable patients who had an increase in CA 19–9, none of the five (0%) underwent an R0 resection compared with 80% of the remaining cohort ($p = 0.001$). Similarly, Aldakkak et al. [65] demonstrated a correlation between post-neoadjuvant CA-19-9 levels and completion of intended therapy including

resection. Katz et al. [66] demonstrated that post-neoadjuvant CA 19–9 < 61 U/mL in upfront resectable patients receiving neoadjuvant chemoradiation had a high positive predictive value for undergoing resection, but a limited negative predictive value.

On the cellular and molecular level, predictive markers are also lacking [67]. Capello [68] and colleagues demonstrated in a comprehensive study that high expression of carboxylesterase 2 might be a predictor of response to FOLFIRINOX, on the basis that carboxylesterase 2 converts irinotecan into its active form, SN-38, which induces apoptosis in PDAC cells. Based on the absence of a reliable marker of resectability as described above, aggressive management of PDACs which have not progressed on neoadjuvant therapy is encouraged. Patients in whom there is no evidence of metastases and a decrease in CA19–9 should be offered a surgical exploration. Determination of resectability should be performed intraoperatively. It is suggested that involved or narrowed vascular structures are examined by serial frozen-section biopsies and that resection is aborted in cases of positive biopsies.

Conclusion

Neoadjuvant therapy may benefit PDAC patients within the whole spectrum of resectability. Patients with locally advanced PDAC may be rendered resectable and offered an operation with a high R0 rate, leading to a survival comparable to upfront resectable patients who offered an operation followed by adjuvant chemotherapy. In borderline resectable PDAC patients, neoadjuvant therapy may increase the frequency of margin-negative resections and improve outcomes. Lastly, in patients presenting with resectable disease, neoadjuvant chemotherapy may aid in selecting surgical candidates by avoiding an operation with high postoperative morbidity and mortality in patients with poor tumor biology. Combination regimens such as FOLFIRINOX and gemcitabine/nab-paclitaxel are preferred over monotherapies in patients who have a good performance status. In the absence of reliable predictors of resectability following neoadjuvant chemotherapy, surgical exploration of all patients who have not disease progression is encouraged. As ongoing neoadjuvant trials for PDAC mature, the impact of neoadjuvant therapy will help us better guide PDAC patients.

Cross-References

► Adjuvant Chemotherapy in Pancreatic Cancer
► Borderline Resectable Pancreatic Cancer
► Chemotherapy for Advanced Pancreatic Cancer

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Differential Therapy Based on Tumor Heterogeneity in Pancreatic Cancer

Juan Iovanna, Benjamin Bian, Martin Bigonnet, and Nelson Dusetti

Contents

Abstract

A major impediment to the effective treatment of patients with pancreatic ductal adenocarcinoma (PDAC) is its molecular heterogeneity, which is reflected in an equally diverse pattern of clinical outcomes and in response to therapies. An efficient strategy in which PDAC samples were collected by endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) or surgery and preserved as patient-derived xenografts (PDX) and as a primary culture of epithelial cells was developed. Multiomics analysis, including transcriptomic and pharmacological studies, was performed on these PDX. As expected, significant molecular and phenotypic heterogeneity was observed. However, bioinformatic analysis was able

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to discriminate between patients with bad or better prognosis. Primary cultures of cells allowed to analyze their relative sensitivity to standard drugs (gemcitabine, 5FU, oxaliplatin, irinotecan active metabolite SN-38, and docetaxel), as well as more original anticancer drugs such as 5-aza-2′-deoxycytidine (5-AZA-dC) or the nicotinamide phosphoribosyltransferase (NAMPT) inhibitor FK866. The establishment of chemograms in vitro allowed to identify individual profiles of drug sensitivity. Remarkably, the response was extremely heterogeneous and patient dependent. It was also found that transcriptome analysis predicts the anticancer drug sensitivity of PDAC cells. Furthermore, an original strategy to identify PDAC dependent on the MYC oncogene and consequently more sensitive to bromodomain and extraterminal inhibitors (BETi) was developed. In conclusion, using this original approach, it was found that multiomics analysis of PDX could predict the clinical outcome of patients, the sensitivity to anticancer drugs, and the pharmacological response to new therapeutic strategies. This opens up a future setting in individualized medicine, aiming to stratify patients in order to select the most appropriate treatments for each group.

Keywords
Individualized Medicine · PDX · Chemograms · Molecular Signatures · Drug Sensitivity · Tumor Heterogeneity

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal human malignancies and a major health problem, causing around 300,000 deaths per year worldwide [1]. Despite considerable research efforts in the past decades, conventional treatment approaches have had limited impact, including surgery, radiation, chemotherapy, or a combination of these. The prognosis is poor with only 20% of patients alive 1 year after diagnosis [1]. Given this scenario, the search for new treatments that will counter PDAC progression and increase patient life expectancy has been given high priority. Particularly, future therapeutic agents are expected to be "molecularly targeted" in order to specifically affect PDAC cells while leaving normal tissues undamaged. Recent phase III clinical trials in unselected PDAC populations tested bevacizumab, erlotinib, and axitinib, which are molecularly targeted agents, combined with gemcitabine. These trials did not show robust survival benefits, probably because they were tested in unselected PDAC populations that were highly heterogeneous [2–4]. In fact, a major impediment to the effective treatment of PDAC is the molecular heterogeneity of the disease, reflected in diverse clinical response patterns to therapy. This heterogeneity is shown by the heterogeneous evolution observed in patients with PDAC, with a survival from 2 to 3 months to more than 5 years after diagnosis, and with a strong difference in susceptibility to classical as well as novel drugs. This may be explained

by the fact that each PDAC has a combination of several modifications to intracellular pathways that will result in variable susceptibility to drugs, metastasis development, and therefore survival [5–7]. Currently, no proposed treatments have taken into account this heterogeneity. In fact, the drugs received by patients suffering from PDAC are chosen according to their general performance status and the stage of their disease. No study of the tumor can predict its responsiveness to the treatment nor give a prognosis to the disease progression. For example, objective response rates of 31.6% in FOLFIRINOX-treated patients and 9.4% in patients treated with gemcitabine have been reported, showing that around 70–90% of patients are non-responders, respectively [8, 9].

Toward a Molecular Pathology Field: Transcriptome of the PDAC is Correlated with Clinical Outcome

Molecular heterogeneity of PDAC has been extensively reported [10–13]. The transcriptome analysis of a cohort of pancreatic PDX revealed a significant correlation between the PDAC phenotype and its clinical outcome [14]. A clustering analysis, using an unsupervised approach, revealed two groups of patients characterized by a bad or a better prognosis. Around 500 transcripts were overexpressed, whereas around 400 transcripts were downregulated in short-term survivors compared with long-term survivors (Fig. 1). Importantly, gene ontology analysis on

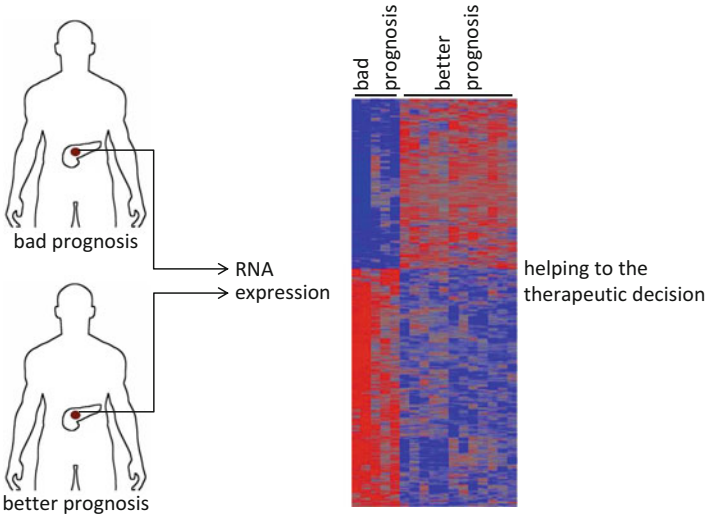

Fig. 1 *RNA expression analysis of PDAC predicts the clinical outcome*. Heat map shows the RNA expression profile of patients with bad and better prognosis

differentially expressed genes showed a significant enrichment in biological processes associated with cancer such as cell cycle, mitosis, response to cellular stresses, DNA metabolism, chromosome organization, and cellular metabolism, with a false discovery rate of >0.001. This indicates that these pathways were preferentially activated [14]. Analysis of clinical data from short-term and long-term survival patients showed that the first group had poorly differentiated tumors whereas long-term survival patients presented partially or well-differentiated tumors. Therefore, these data are not surprising since poorly differentiated tumors are expected to be associated with bad prognosis compared with well-differentiated tumors [15–18]. This correlation between tumor differentiation and prognosis has been previously documented. Wasif et al. described a correlation between tumor differentiation and patient survival time, as well as using tumor differentiation as a value to predict response to treatment [19]. In fact, tumor differentiation, or "grade," is increasingly used as an independent prognostic factor; it appears with as much impact as prognostic tumor size or lymph node metastatic invasion [20]. Although the grade of differentiation can be estimated by the pathologist after a pancreatectomy, this is only possible in about 15% of patients. However, using a set of molecular markers identified in recent works, the grade of the PDAC could be estimated in only a small number of cells obtained by endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA). This is almost impossible to determine by microscopy analysis alone on these small samples. In fact, based on these results, it seems evident that using expression of some of these molecular indicators on small PDAC samples obtained by EUS-FNA would help in predicting the behavior of the PDAC and shape the therapeutic strategy. In theory, the microscopic analysis of small PDAC samples could be replaced by a molecular analysis combined with nanotechnologies, e.g., NanoString. Although this novel approach is promising, it must be validated in independent cohorts of patients.

The PDAC Phenotype is Associated with Chemosensitivity

In a recent study, the sensitivity of PDAC primary cell lines derived from PDX to five gold standard chemotherapies (gemcitabine, 5FU, oxaliplatin, docetaxel, and the irinotecan active metabolite named SN-38) was analyzed, with drug concentrations ranging from 0.001 to 1000 μM. These personalized chemograms allowed to obtain a dose-response curve characterizing each patient [14]. These results demonstrated that each patient-derived cell line shows its own chemogram profile, indicating that each PDAC has a particular and specific profile of response (Fig. 2). This is clinically relevant since sensitivity or resistance to one drug does not predict sensitivity or resistance to another. Another important point to be noted is that after incubation with some drugs, it was almost impossible to kill all the cells even with very high concentrations such as 1000 μM for gemcitabine, oxaliplatin, docetaxel, or 5FU or 100 μM for SN-38. For example, 20–50% of cells were resistant to 1000 μM of gemcitabine, 5–30% remained alive when treated with 100 μM of SN-38, 10–70%

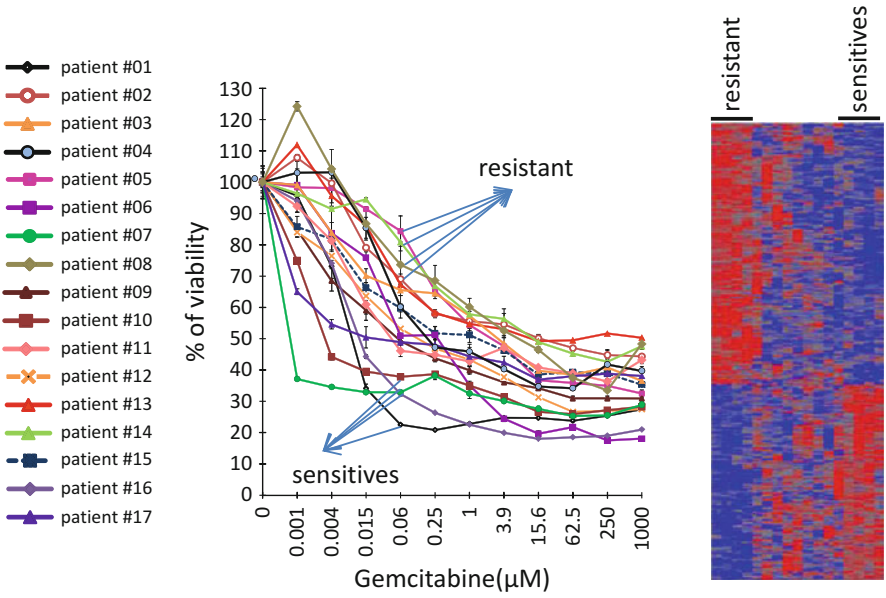

Fig. 2 *Chemograms*. PDAC-derived cells were treated with increasing concentrations of gemcitabine, and the surviving cells were measured after 72 h of treatment. A sensitivity profile was obtained for each patient. *RNA expression and drug sensitivity*. The heat map is showing the RNA expression profiles of PDAC-derived cells with resistance or sensitivity to the treatment with gemcitabine

when treated with 1000 μM 5FU, and 0–25% with 1000 μM oxaliplatin. An exception was found when cells were treated with docetaxel, where doses as low as 0.001 μM were able to kill from 20% to 90%, depending on the primary culture, and almost all cells were killed by 62.5 μM. This observation can be explained by the fact that primary cultures are representative of the different cell populations present in the tumor, as PDAC is known to be heterogeneous [21]. This emphasizes clinical applicability because the chemogram may detect the percentage of sensitive and resistant cells to a drug and therefore be a helpful tool to the oncologist selecting the second line of treatment for a given patient.

Then it was studied the correlation between drug response and PDAC phenotype by performing a clustering analysis of each PDX transcriptome (Fig. 2). Surprisingly, some sets of genes were identified as specifically overexpressed or underexpressed in resistant and in sensitive cells, respectively [14]. Importantly, it was observed that a small number of common genes associated with drug resistance or sensitivity, suggesting that the phenotype of the sensitivity or resistance is specific for each drug [14]. Finally, it was noted that the genes associated with sensitivity or resistance to treatment are different to the genes associated with bad or better prognosis. This indicates that survival and drug sensitivity are regulated by independent mechanisms [14].

Repositioning Unusual Anticancer Drugs for Treating a Selected Subgroup of Patients with PDAC

The Example of 5-AZA-dC

5-AZA-dC (5-aza-2′-deoxycytidine) is a DNA methyltransferase (DNMT) inhibitor incorporated into DNA as a deoxycytidine analogue, forming irreversible covalent bonds with DNMT at cytosine sites targeted for methylation [22]. 5-AZA-dC demonstrates activity against hematological malignancy [23] and is used as the first line of treatment in acute myeloid leukemia patients over 65 years old who are not candidates for intensive chemotherapy [24]. However, its efficacy in solid tumors seems to be limited [25]. The rationale to use methyltransferase inhibitors to treat tumors is that neoplastic cells exhibit global hypomethylation with localized hypermethylation of CpG islands and increased levels of methyltransferases activity [26]. Moreover, aberrant hypermethylation of CpG islands is associated with transcriptional silencing of genes, which not only plays a role in tumorigenesis but may also influence response to anticancer agents [27, 28]. Therefore, reversing gene methylation and epigenetic silencing has the potential to influence tumor growth, sensitivity to anticancer agents, and ultimately clinical outcome [29]. Several studies have documented the relevance of epigenetic alterations in pancreatic cancer and the effect of 5-AZA-dC on pancreatic tumor cells [30]. In clinical trials, although the 5-AZA-dC has shown an objective response in some patients, its overall efficacy remains relatively low. For these reasons, 5-AZA-dC is not used in the treatment of patients with PDAC. Therefore, this drug has been selected as a proof of concept to study whether a drug with a relevant mode of action is efficacious in a particular subgroup of PDAC patients and whether that group can be identified by specific markers. Several primary cultures of PDAC cancer cells were subjected to increasing concentrations (from 0 to 80 μM) of 5-AZA-dC in order to study their sensitivity and to obtain a dose-response curve. Using this approach, it was possible to compare these PDAC-derived primary cultures and estimate their relative chemosensitivity. As with the gold standard anticancer drugs, each patient-derived primary culture showed a different pattern of chemosensitivity with an IC_{50} ranging from 0.29 μM to $>$80 μM, which is a range of more than 275-fold. Then their relative sensitivity in vivo using pancreatic PDX was validated [31].

This strong variability in response to the drug encouraged to go forward with this study, trying to find molecular markers that may identify sensitive patients. Surprisingly, there is no correlation between sensitivity to 5-AZA-dC and DNMT1, DNMT3A, or DNMT3B at the expression level. In addition, expression of other molecules associated with DNA methylation, such as Mecp2 (methyl-CpG-binding protein 2) or polycomb-group proteins including SUZ12, EED, EZH1, and EZH2, does not correlate with the 5-AZA-dC sensitivity. These results are interesting and original because they show that the effect of the drug is not systematically dependent on the level of its target, indicating that sensitivity is dependent on other cellular mechanisms. This is reflected in the lack of literature associating efficacy of 5-AZA-dC and levels of DNMT1 expression in tumors, with the exception of data

obtained by Li et al. This concluded that PDAC-derived cells with low DNMT1 expression tend to be sensitive to low doses of 5-AZA-dC [32]. Altogether, these results strongly suggest that there is little to no correlation with their targets. However, it has been found that sensitivity to 5-AZA-dC treatment does correlate with long-term survival in patients carrying well- and moderately differentiated tumors. This is in agreement with the fact that some genes typically expressed in poorly differentiated PDAC, such as MUC3A, MUC5AC, GATA6, or HNF4A, are differentially overexpressed in sensitive PDAC-derived cells compared with resistant PDAC-derived cells. These data strongly suggest that 5-AZA-dC treatment should be more efficient against well- and moderately differentiated tumors than against the poorly differentiated ones.

The Example of the NAMPT Inhibitor FK866

Nicotinamide phosphoribosyltransferase (NAMPT) catalyzes the rate-limiting step of nicotinamide condensation with 5-phosphoribosyl-1-pyrophosphate to yield nicotinamide mononucleotide and is overexpressed in several tumors. FK866 ([3,5-bis(trifluoromethyl)phenyl][(2R)-2-(3-hydroxy-4-methylbenzyl)-4-{2-[(2S)-2-(methoxymethyl)morpholin-4-yl]ethyl}piperazin-1-yl]methanone dihydrochloride) is a noncompetitive highly specific inhibitor of NAMPT and is clinically interesting as it is a potent antitumor drug both in vitro and in vivo [33]. Many recent studies provide evidence that it selectively inhibits growth of various types of cancer cells, with no effect on normal cells [34]. It causes cellular death by apoptosis [35] and induces autophagy. Clinical studies have revealed that FK866 induces toxicity to proliferating hematopoietic cells, due to its short half-life in circulation and the resulting prolonged treatment regimens. Therefore, the efficiency of NAD+ (nicotinamide adenine dinucleotide)-depleting drugs, such as NAMPT inhibitors, when used alone is expected to be low due to insufficient tumor selectivity [36–38]. For this reason, FK866 has mainly been tested as an additive drug to other well-known chemotherapies. It increased the chemosensitivity of gastric cancer cells to 5FU [39], potentiated the effects of cisplatin and etoposide in neuroblastoma cell lines [40], and massively reduced the overall metabolic activity in xenografts, impairing PDAC growth [41].

It was studied the effect of the NAMPT-inhibitor FK866 in PDAC-derived cells. Primary cultures of PDAC-derived cells were exposed to increasing concentrations of FK866 (from 0 to 1000 nM) to determine their sensitivity by plotting dose-response curves. Using this approach, it was estimated the relative chemosensitivity of the different PDAC-derived cell cultures by comparing the resulting IC_{50} values. These data reveal that each PDAC-derived cell culture has its own sensitivity to FK866 with a huge range of IC_{50} values (from 0.30 to >1000 nM), suggesting a very high response variability among patients [42]. This has also been described for the gold standard drugs and 5-AZA-dC. Next, it was hypothesized that NAMPT level, as a specific target of FK866, could predict drug sensitivity. Consequently, it was quantified NAMPT at the transcriptional level in pancreatic PDX. The mRNA

expression level was then plotted, and the FK866 IC_{50} values were used to investigate and compare the global sensitivity of PDAC cells. These results showed that resistance to FK866 positively correlates with the expression level of NAMPT transcript indicating that PDAC expressing higher levels of NAMPT has an increased resistance to FK866 treatment, possibly for a stoichiometric reason.

It is unlikely that NAMPT inhibition could be used as a monotherapy for treating patients with a PDAC since FK866 at high concentrations is very toxic, due to its mechanism of action affecting basic functions of both cancerous and normal cells. Therefore, the only possibility is to use FK866 in combination with cytotoxic drugs to potentiate their effect. Consequently, it was studied the sensitivity of PDAC to the treatments with gemcitabine alone or gemcitabine combined with FK866 in several PDAC-derived primary cultures [42]. The combined treatment (gemcitabine + FK866) synergistically decreased the cell viability of 70% of the primary cultures compared with treatment with gemcitabine alone. Surprisingly, this added benefit was almost negligible when combined with 5FU or oxaliplatin [42]. Then it was analyzed the effect of FK866 alone or combined with gemcitabine on the intracellular levels of NAD+ and found a significant correlation between low levels of NAD + in pancreatic PDX and its sensitivity to the treatment. Overall, these results suggest that most PDAC patients could take advantage of co-treatment with gemcitabine + FK866. In addition, quantification of NAMPT mRNA expression or NAD+ concentration in PDAC could be used as potential biomarkers for determining their sensitivity to the co-treatment of gemcitabine + FK866.

Identifying Novel Personalized Targets for Treating Patients with PDAC

Like other malignant diseases, PDAC results from a complex combination of genetic, epigenetic, and environmental factors, which gives rise to a particularly heterogeneous disease [43–45]. Consequently, this heterogeneity highlights the need to stratify patients with the goal of predicting better responses to therapies. One strategy to discover potential markers for patient stratification is to focus on identifying pathways that are deregulated in tumors, particularly when tumor cell survival depends on keeping these alterations (e.g., oncogene "dependence" to survive and grow) [46, 47]. Therefore, it is logical to assume that targeting of these pathways with specific inhibitors, when available, should lead to cell growth arrest, death, and tumor regression. Using this rationale, it should be possible to select, by means of a few markers, a particular subgroup of patients whose tumor cells are "addicted" to certain pathways, and use appropriate inhibitors to treat these patients' tumors, which is the major goal of modern individualized medicine.

In this way, a frequently deregulated, though insufficiently therapeutically exploited, pathway in PDAC involves "dependence" on the *c-Myc* oncogene [48]. This transcription factor influences the expression of a significant number of genes involved in cell growth, proliferation, and apoptosis [49–52]. In fact, this oncogene has been implicated in the pathogenesis of one-third of all human malignancies.

Early studies confirmed the oncogenic role of c-MYC in PDAC using genetically engineered mouse models, which upon overexpression of this gene display increased pancreatic tumorigenesis [53]. In addition, using a variety of experimental models, it has been shown that upregulation of MYC is sufficient to induce the formation of PDAC without additional genetic manipulation of any cell survival pathways [54], and deletion of one *c-Myc* allele decelerates tumor development in vivo [55]. Based on these data, in recent work, Wirth et al. propose to use MYC as a stratification marker of PDAC [56]. Altogether, these features indicate that *c-Myc* behaves as a cancer driver gene for PDAC. Consequently, many efforts have been dedicated to identify potent MYC inhibitors as new therapeutic options [57–60]. Key to these efforts has been the discovery that the bromodomain and extraterminal family of proteins (BET) are necessary for MYC activity [61, 62]. These proteins are efficiently inhibited by BET inhibitors (BETi), such as JQ1, suppressing PDAC development in mice by inhibiting both MYC activity and inflammatory signals [63]. In addition, inhibition of MYC expression is thought to be an essential mechanism by which BETi suppress tumor progression [64–66]. Thus, identifying a subgroup of PDAC patients based on their c-MYC-high status and testing their response to BETi could be of paramount medical importance. Consequently, this hypothesis was recently tested [67].

To this end, a learning cohort of 55 pancreatic PDX was created and characterized and gene expression profiling performed using an Affymetrix platform. From this dataset, a panel of 239 RNAs known to be regulated by MYC was selected and performed a hierarchical clustering analysis. The obtained dendrogram indicates the presence of two major subgroups that were logically defined as MYC high and MYC low. Interestingly, it was observed that around of 30% of patients are characterized by an increase in the expression of MYC target RNAs. In addition, it was also found that the tumors of the MYC-high subgroup showed lower differentiation, proliferated more, and presented a shorter survival time (median is 9.2 months for the MYC-high subgroup vs. 18.8 months for the MYC-low subgroup). Moreover, the relapse-free survival median is 5.6 months and 11.5 months for MYC-high and MYC-low subgroups, respectively. These results indicate that PDAC of the MYC-high subgroup is more aggressive. To optimize the response to BETi treatment, a minimal specific MYC signature was defined to be used to stratify PDAC tumors as MYC high or MYC low. A total of 16 genes were selected, 10 were identified from the gene set corresponding to the upregulated genes in the MYC-high group, and 6 were the top-scoring downregulated genes in the MYC-high patients, found from the whole gene expression profile. An algorithm was developed by using the ratio of both up- and downregulated genes to identify PDAC tumors with either MYC-high or MYC-low phenotypes (Fig. 3). The accuracy of this algorithm was confirmed using an independent validation cohort. Therefore, it was concluded that the algorithm based on these small numbers of transcripts is reliable for identifying tumor subtypes based on their c-MYC status.

Accordingly, it has been assumed that the subgroup of PDAC belonging to the MYC-high phenotype should be more sensitive to the pharmacological inhibition of MYC activity. This currently cannot be targeted directly but instead is targeted

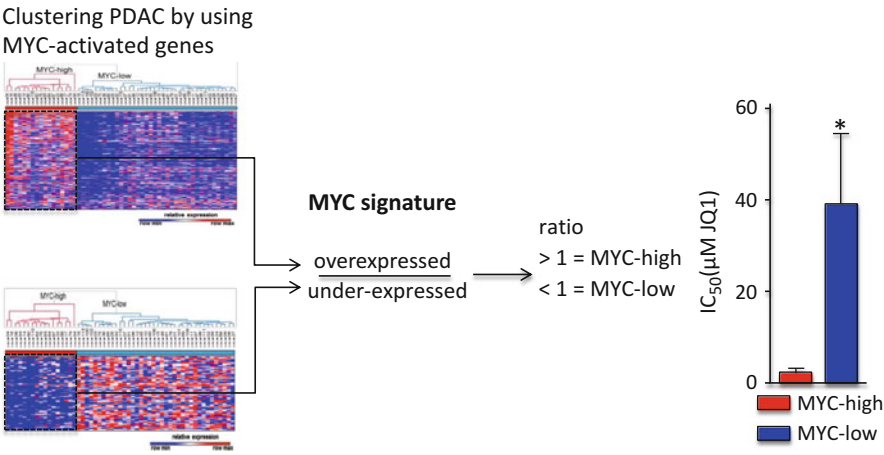

Fig. 3 *MYC signatures predict sensitivity to BETi*. MYC controls the expression of a large panel of target genes that characterize tumor phenotypes. A 16-transcript signature classifies a MYC-dependent (MYC high) PDAC subgroup by performing the ratio of overexpressed over underexpressed genes after normalization. Ratio > 1 indicates a MYC-high profile, and ratio < 1 corresponds to MYC-low profile. MYC-high tumors are more sensitive to JQ1 treatment, a well-described bromodomain inhibitor

through the inactivation of BET proteins. To test the hypothesis, a panel of pancreatic PDX-derived primary cultures has been treated with the well-characterized BETi compound JQ1. Cells were incubated with a large range of drug concentrations, and it has been found that MYC-high cells exhibit higher sensitivity to the BETi treatment compared with the MYC-low cells. The mean IC_{50} for the MYC-high cells was 2.3 µM $\pm$ 0.8, whereas the corresponding IC_{50} for the MYC-low cells was 39.22 µM $\pm$ 16. Then, a preclinical analysis was performed by treating PDX presenting MYC-high or MYC-low phenotypes with JQ1 to validate the in vitro results. As expected, MYC-high samples responded efficiently to the treatment, whereas samples with the MYC-low phenotype were more resistant. Altogether, from the in vitro and in vivo results, it can be assumed that MYC-high tumors are more sensitive to BETi. The main conclusion is that having tools to determine tumors with high MYC activity is of clinical interest in order to identify patients sensitive to BETi. These results also suggest that a similar strategy may be useful in designing individualized medicine efforts aimed at stratifying patients to novel treatments.

Conclusion

Determination of efficient molecular signatures is clinically useful for detecting patients having a particular pattern of sensitivity to a given treatment. The approaches presented here are easily applicable and low cost. This is particularly

beneficial in nonoperable tumors, which represent around 85% of PDAC. Currently, in these patients, a biopsy is systematically taken by EUS-FNA as a diagnosis confirmation procedure prior to treatment. These biopsies represent a valuable source of cancer cells, which may serve as the source of tumor macromolecules such as RNA. In turn, this RNA may be used for measuring expression of RNA sets of interest (expression signatures) to determine a particular phenotype. Unfortunately, one of the main difficulties found with biopsies is that they are systematically contaminated by blood, stroma, and, in some cases, normal pancreatic or gastrointestinal cells, which may make molecular analyses difficult. The preparation of PDX from biopsies in order to obtain sufficient clean material is technically feasible, but it would take nearly 6 months. This delay is incompatible with clinical application. The alternative is to prepare organoids directly from biopsies. This allows amplification by cell replication and purity because only epithelial cancerous cells will grow in the selective culture media. It is possible to obtain suitable material within 2–3 weeks of culture. Then, it is easy to purify RNA from organoids and measure expression of several informative transcripts using the NanoString platform (Fig. 4). This approach of transcriptional level quantification presents a great advantage in that it does not require previous amplification, which can introduce unwanted

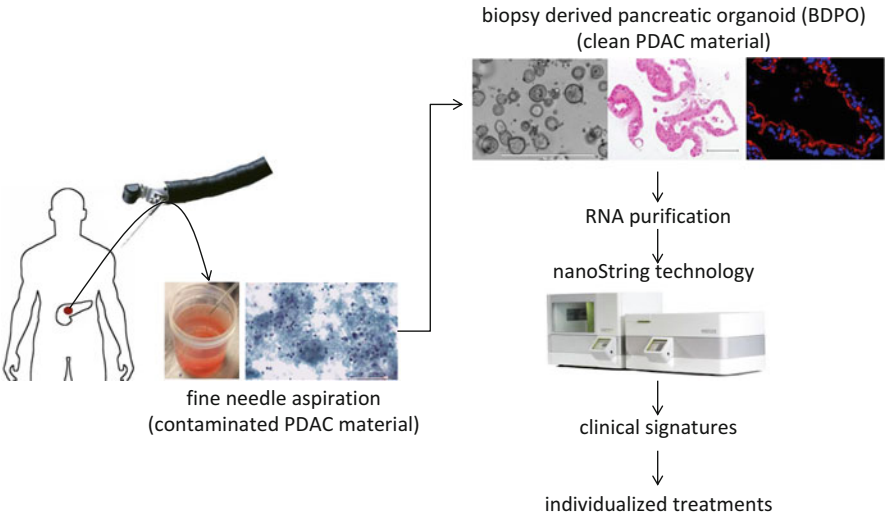

Fig. 4 *Molecular signatures of PDAC tumors will be routinely performed for individualized treatment approaches in the near future*. It will be of particular interest for non-operable patients which represent about 85% of PDAC. Currently, in these patients, a biopsy is systematically taken by EUS-FNA before starting the antitumor treatment as a diagnosis confirmation procedure. These biopsies represent a valuable source of cancer cells which may serve as the source of RNA. But the material obtained is largely contaminated by blood and tumor stroma or neighbor tissues. The alternative is to prepare organoids directly from biopsies that allows amplification by cell replication and purity since only epithelial cancerous cells are growing in the selective culture media. Sufficient organoids can be obtained within 2–3 weeks of culture. RNA from organoids can be easily purified and expression of several informative transcripts measured by a NanoString platform

technical bias. Importantly, all these manipulations take only 3 additional days. It is very probable that in the near future, the treatment of cancer will be preceded by a precise and extensive molecular characterization of cancer cells in order to select the most appropriate treatments, creating an individualized medicine approach. PDAC is undoubtedly one of the malignant diseases that most urgently needs this approach, since treatment with standard drugs is inefficient. Although this chapter was focused on PDAC, a similar strategy could also be applied to other cancer types.

Cross-References

- ▶ Chemotherapy for Advanced Pancreatic Cancer
- ▶ Clinical Decision-Making in Pancreatic Cancer
- ▶ Development of Novel Therapeutic Response Biomarkers
- ▶ Diagnostic Biomarkers
- ▶ Differential Therapy Based on Tumor Heterogeneity in Pancreatic Cancer
- ▶ Emerging Therapeutic Targets in Pancreatic Adenocarcinoma

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Neoadjuvant Chemoradiation for Operable Pancreatic Cancer: The Importance of Local Disease Control

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Contents

Abstract

Pancreatic cancer (PC) is one of the most neuroinvasive tumors of the gastrointestinal tract, and perineural invasion is associated with high rates of local-regional

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recurrence. Historically, the goal of local-regional control in patients with PC has largely been secondary to the prevention of metastatic disease progression. However, with improving systemic therapies, patients are now experiencing unprecedented survivals and are living long enough to be susceptible to local recurrence. Such local recurrences usually occur in the neural tissue enveloping the celiac artery, superior mesenteric artery, or hepatic artery. The use of neoadjuvant chemoradiation has been effective in decreasing perineural invasion and may be particularly effective when given preoperatively prior to the immune suppressive effects of surgery and the hypoxic tissue disruption that occurs following pancreatectomy. This chapter focuses on the rationale and importance of neoadjuvant radiation therapy in the treatment of localized, potentially operable PC and provides an introduction to current neoadjuvant radiation therapy techniques, including intensity modulated radiation therapy and stereotactic body radiation therapy.

Keywords
Radiation therapy · Neoadjuvant · Perineural invasion · Intensity modulated radiation · Stereotactic body radiation

Introduction

Optimal treatment sequencing for patients with localized, operable pancreatic cancer (PC) is the subject of intense investigation as it is now appreciated that almost all patients have radiographically occult metastatic disease at the time of diagnosis [1]. Multimodality therapy has become the standard; for example, in patients treated with a surgery-first approach, all current guidelines recommend 6 months of postoperative (adjuvant) therapy, regardless of final pathologic stage [2]. Unfortunately, the delivery of adjuvant therapy following pancreatectomy is unpredictable, as approximately 40–60% of patients will not receive adjuvant therapy due to perioperative morbidity or failure to adequately recover from surgery [3, 4]. The inability to deliver adjuvant therapy to patients with a high probability of harboring micrometastatic disease has fueled an interest in alternative treatment sequencing. Preoperative (neoadjuvant) therapy is a logical alternative to surgery-first treatment sequencing. Inherent to a neoadjuvant approach is the immediate delivery of systemic therapy to a population of patients at high risk of harboring disease outside of the primary pancreatic tumor. It also allows for a 3–5 month period of treatment during which patients will evidence response or progression [5]. Among patients who complete all intended neoadjuvant therapy and surgery, the median overall survival has been reported to range from 34 to 45 months, suggesting that early delivery of systemic therapy prior to surgery may be even more effective than adjuvant therapy [6, 7]. The timing or sequencing of systemic therapy in operable patients may have oncologic value beyond just the receipt of systemic therapy. As survival duration increases, local disease control will become even more important as patients will live long enough to be susceptible to local recurrence. An obvious

clinical reality is that only patients who are alive and free from distant metastatic disease-related death are prone to local recurrence. The short survival experienced by most patients after a potentially curable operation for PC, due to early metastatic disease progression, has made assessment of local disease control impossible. Isolated local recurrences (after pancreatic resection) virtually always arise within the perineurium of the autonomic nerves which surround the celiac, hepatic, or superior mesenteric arteries. Such recurrences are difficult to treat and prevention is the preferred strategy. This chapter will focus on the pathophysiology of local perineural recurrence and the importance of radiation therapy in its prevention.

Brief Update on Pretreatment Staging

Historically, resectability was determined at the time of operation for patients with localized PC; if the surgeon felt the tumor was resectable, the tumor was removed and the patient was declared to have had resectable disease. If at the time of operation the tumor was not felt to be resectable, the patient was declared to have locally advanced disease. Subsequently, an objective CT-based staging system was developed to improve the classification of patients eligible for neoadjuvant clinical trials, where a preoperative definition of resectable disease was needed to identify eligible patients for trial enrollment [8]. The benefit of such an objectively defined staging system for patients and physicians is obvious – the goals of therapy can be specifically defined at the time of diagnosis and optimal treatment sequencing can be initiated. To the extent that surgery is necessary (albeit usually not sufficient) for cure, patients who may be eligible for potentially curative surgery can be accurately defined; and those patients with locally advanced (nonoperable) disease are also identified. Historically, among patients who have locally advanced PC as defined by preoperative imaging, surgery was not felt to be possible. However, it soon became clear that a gray-zone existed between the definitions of resectable and locally advanced PC. Borderline resectable disease was used to define patients with arterial abutment and short segment venous (superior mesenteric–portal vein [SMV-PV]) occlusion who, in the past, would have been considered locally advanced [9]. However, after neoadjuvant therapy the borderline classification was developed for patients who demonstrated a response to treatment, as measured by clinical benefit, improved imaging, and a decline in tumor marker profile, and were being considered for surgery [10]. Patients with borderline resectable PC are at the highest possible risk for a positive margin of resection due to tumor-artery abutment, require a more complex operation usually involving vascular resection and reconstruction, and may be at higher risk for harboring radiographically occult distant metastatic disease. For these reasons, at the Medical College of Wisconsin (MCW), induction therapy consisting of chemotherapy followed by chemoradiation is the preferred treatment sequence in this patient population. The chemoradiation portion of induction therapy is thought to be particularly important for those patients with

arterial abutment in the hope of sterilizing at least the periphery of the tumor and thereby preventing a positive margin of resection.

The staging system used for clinical trial enrollment at MCW is illustrated in Table 1 and incorporates an expanded description of locally advanced disease which

Table 1 Staging classification of localized PC

Vascular structures which determine the stage of disease for localized pancreatic cancer		Resectable	Borderline resectable	Locally advanced	
				Type A	Type B
Tumor-artery anatomy	SMA (usually pertains to a tumor of the head or uncinate process)	No radiographic evidence of abutment or encasement	≤180° (abutment)	>180° (encasement) but ≤270°	>270° encasement
	Celiac artery (usually pertains to a tumor of the pancreatic body)	No radiographic evidence of abutment or encasement	≤180° (abutment)	>180° (encasement) but does not extend to the aorta and amenable to celiac resection (with or without reconstruction)	>180° and abutment/ encasement of the aorta
	Hepatic Artery (HA) (usually pertains to a tumor of the pancreatic neck/head)	No radiographic evidence of abutment or encasement	Short segment abutment/ encasement without extension to celiac artery or HA bifurcation	>180° encasement with extension to celiac artery and amenable to vascular reconstruction	>180° encasement with extension beyond bifurcation of proper HA into right and left hepatic arteries
Tumor-vein anatomy	SMV-PV	≤50% narrowing of SMV, PV, SMV/PV	>50% narrowing of SMV, PV, SMV/PV **with** a distal and proximal target for reconstruction	Occlusion **without** option for reconstruction	
Traditionally considered for resection after neoadjuvant therapy		**Yes**	**Yes**	**Yes**	**No**

Abbreviations: *SMA*, superior mesenteric artery; *SMV*, superior mesenteric vein; *PV*, portal vein; or *SMV-PV*, superior mesenteric-portal vein; *CHA*, common hepatic artery; *NA*, not applicable

has been termed Type A and Type B [11]. Patients with Type A locally advanced disease may be candidates for surgical resection of their tumor after induction therapy, whereas surgery will likely never be possible in those patients with Type B disease. Because the visceral arteries have a perineural sheath which envelopes them, there is often a plane of dissection between the adventitia of the artery and the neural sheath which allows for sharp dissection of the tumor off the artery. In contrast, complete 360° encasement would require that one cuts through tumor to separate the superior mesenteric artery (SMA) from the tumor, SMA encasement of this magnitude is considered nonoperable at MCW. With regard to the celiac artery, increasing experience has demonstrated the safety of celiac resection in carefully selected patients with tumors of the pancreatic body which have responded to induction therapy [12]. The threshold for considering surgery following induction therapy in patients with locally advanced PC is evolving. However, it is important to note that the expanded use of surgery in very highly selected patients is guided by an objective, reproducible pretreatment and preoperative CT-based staging system (Table 1).

Perineural Invasion in Pancreatic Cancer

Since the pancreas is a retroperitoneal organ which is adjacent to major vascular structures including the SMA and celiac trunk, PCs which abut or encase these vessels often infiltrate. The pancreas is richly innervated by the adjacent celiac and superior mesenteric nerve plexuses, and the close proximity of a PC to both intra- and extrapancreatic nerves allows for the direct infiltration of cancer cells into nerves and the dissociation of cells away from the primary tumor [13]. This process of cancer infiltration into neural tissue is called perineural invasion (PNI) and PNI is a risk factor for local disease recurrence. PNI has been observed in as many as 80–100% of resected PC specimens, making PC one of the most aggressive and neuroinvasive gastrointestinal malignancies [13–18]. In a study of 90 patients with resected PC by Takahashi et al., 88 (98%) patients had intrapancreatic PNI and 47 (52%) had both intra- and extrapancreatic PNI within the retroperitoneal perivascular neural tissues along the SMA, common hepatic artery (CHA), and aorta [16]. Extrapancreatic PNI was identified as a poor prognostic factor associated with decreased overall survival. This finding has been corroborated by a recent meta-analysis, including 121 studies, which identified a 1.68-fold increased risk of death with the presence of PNI (95% CI: 1.47–1.92; $p < 0.00001$) [15]. Importantly, PNI was also associated with a 2.53-fold increased risk of disease progression (95% CI: 1.67–3.83; $p = 0.0001$).

The high incidence of PNI in PC may not be related purely to anatomic considerations, as interestingly, the presence of PNI is independent of tumor size and location, suggesting that additional factors may promote the pathogenesis of PNI [16]. Peripheral nerve Schwann cells are present in precursor lesions of PC in both human PC and genetically engineered mouse models, and the frequency of Schwann cells in the precursor lesions has been correlated with the frequency of neural

invasion in PCs [19]. Furthermore, studies have shown that cancer associated fibroblasts promote the migration of peripheral nerve Schwann cells through Cadherin-2 (neural cadherin) and beta-catenin signaling [20]. In addition, murine models have demonstrated the secretion of glial cell line-derived neurotrophic factor by nerve cells has a direct chemotactic effect on PC cells, resulting in the directional migration of cancer cells towards nerves, and subsequently invasion of nerves by cancer cells [21, 22]. These findings suggest that neural tissue may be recruited by the tumor as an early event in carcinogenesis rather than neural infiltration occurring as a late event of cancer metastases.

Given the ubiquitous presence of PNI and its high association with local recurrence, strategies which enhance local-regional control are likely to be important in the management of PC. Even among patients who successfully undergo pancreatic resection, the local failure rate has been reported to be as high 80% [23]. In more contemporary series of patients who underwent a surgery-first approach, local recurrence rates have been reported to be 24–45% [23, 24]. Although the rationale for neoadjuvant therapy in patients with PC was motivated by a desire to both detect and treat micrometastatic disease prior to surgery, unexpectedly, neoadjuvant therapy, particularly neoadjuvant chemoradiation, has also been associated with superior local-regional disease control. Such improved local control has been associated with lower rates of positive margins, lymph node metastases, and PNI observed in the posttreatment pathologic specimens [25–27]. Neoadjuvant therapy results in decreased rates of PNI as compared to rates observed with a surgery-first approach [18, 26, 28]. For example, in a study by Ferrone et al. evaluating the benefit of neoadjuvant FOLFIRINOX among patients with borderline resectable or locally advanced PC, PNI was identified in 29 (72.5%) of 40 patients treated with neoadjuvant FOLFIRINOX with or without radiation as compared to 83 (95.4%) of 87 treated with a surgery-first approach [18]. Similarly, Chatterjee et al. observed PNI in 123 (58%) of 212 patients treated with neoadjuvant chemoradiation as compared to 48 (80%) of 60 patients who were treated with a surgery-first approach. Among the 212 patients who received neoadjuvant chemoradiation, the presence of PNI was associated with a significant decrease in disease-free survival (11 months with PNI vs. 22 months without) and overall survival (28 months with PNI vs. 56 months without) [17].

Although not completely understood, current data suggests the mechanism by which chemoradiation decreases PNI in pancreatic tumors may be a twofold process. In general, the delivery of radiosensitizing chemotherapy with concurrent radiation is effective at inducing cell death, thereby decreasing the number of cancer cells along the intrapancreatic nerves. However, there is data suggesting that radiation may specifically alter the nerve microenvironment resulting in less PNI [29]. In an in vivo murine model, PC cells were injected into the surgically exposed sciatic nerves of mice. Utilizing both magnetic resonance imaging (MRI) and hematoxylin and eosin (H&E) staining, the investigators observed more extensive PNI in the

sciatic nerves of the nonradiated mice as compared to radiated mice [29]. Further, the delivery of radiation resulted in the suppression of glial cell line-derived neutrophic factor secretion by nerve cells, which is known to have a chemotactic effect on PC cells. The mean concentration of glial-derived neutrophic factor secreted from sciatic nerves was reduced to 65 pg/mL from 130 pg/mL following a single dose of 8 Gy [29].

Are Local Recurrences Preventable with Appropriate Treatment Sequencing?

Local recurrences are a major cause of morbidity and mortality among patients with resected PC, and arguably, they may be preventable. In series of patients treated with surgery first, isolated local recurrence as the first site of recurrence is reported in 20–60% of patients, and as many as 80% will have developed recurrent local disease by the time of death – powerful data in support of the critical need for effective local-regional therapies [24, 30]. A rationale for neoadjuvant chemoradiation is to enhance sterilization of any local-regional micrometastatic disease prior to surgery, thereby reducing the probability of residual microscopic disease which can serve as the nidus for local treatment failure. Neoadjuvant chemoradiation has proven to be effective at achieving local-regional disease control, and this has resulted in a remarkable decrease in the incidence of local recurrences. One of the first trials to demonstrate the benefit of neoadjuvant chemoradiation was a phase II clinical trial performed at M.D. Anderson Center which evaluated the efficacy of neoadjuvant gemcitabine-based chemoradiation among 86 patients with localized PC of which 64 (74%) patients completed all neoadjuvant chemoradiation and surgery. The median time to progression among all 64 resected patients was 28.6 months from diagnosis, and local recurrences developed in 7 (11%) patients [6]. In another cohort of 69 patients with resectable or borderline resectable PC, treated with neoadjuvant intensity modulated radiation therapy (IMRT), the local recurrence rate was 5 (7%) [26]. The significant decrease in local recurrence rates suggests the delivery of chemoradiation prior to surgical intervention may be a highly effective strategy to treat the occult local-regional micrometastatic disease.

Lastly, it is important to note that most local recurrences develop within millimeters of the SMA and celiac artery, as these vessels are immediately adjacent to a surgeon-created margin and PCs frequently extend along the perivascular neural tissues [24]. Although meticulous surgical technique may allow for the dissection of tumor away from the adventitia of the artery, over 40% of patients will have residual tumor cells at the resection margin, which often remain undetected [31]. In a report by Katz et al. involving 194 patients with localized PC of which 147 (76%) received neoadjuvant chemoradiation, the investigators observed the delivery of neoadjuvant chemoradiation was associated with an increased SMA margin distance (>1 mm) and this was associated with a decreased incidence of local recurrence. The median

time to disease recurrence was 19.5 months for all patients, and isolated local-regional recurrence occurred in 14%, isolated distant in 37%, and concurrent local and distant in 9% of patients [32]. This study highlights the importance of neo-adjuvant chemoradiation and meticulous surgical technique, as both may be necessary to achieve local disease control and minimize the rate of local recurrences.

Evolution of Neoadjuvant Chemoradiation for PC

Radiation to the upper abdomen must be delivered with careful planning and great accuracy to adeptly irradiate the defined pancreatic tumor volumes while partially avoiding the many normal organs which live near the pancreas. Significant progress has been made in defining the treatment targets and shaping the dose distribution to securely cover the areas that need radiation and partially avoid the normal adjacent structures. Advanced multi-planar imaging used for radiation planning, including CT, MRI and PET scans, enables excellent target definition, selective dose escalation to key parts of the target volume, and reduction of the irradiated volumes in the sensitive upper abdomen. This leads to better patient tolerance and reduced intraoperative complications if used in the neoadjuvant setting [33]. Use of three dimensional image-based conformal radiotherapy (3DCRT) has been closely followed by development of IMRT.

Intensity Modulated Radiation Therapy (IMRT) for Pancreas Cancer: Neoadjuvant Approaches

IMRT is an advanced version of 3DCRT that entails use of sophisticated computer controlled radiation beam delivery by varying beam intensities within each beam portal to improve the conformity of the dose distribution to the shape of the tumor with associated avoidance of adjacent normal organs. IMRT treatment planning is performed using inverse treatment planning where the planning target volume (PTV) dose is specified as well as the allowable doses/volumes to the adjacent normal organs. The computer program then calculates a customized intensity pattern to best meet the specified dose volume constraints for the PTV and normal organs. In addition to accurate target definition, image guided radiation therapy is the process of positioning the patient on the treatment table and using on board imaging to localize the tumor and adjacent organs at risk before each delivered radiation treatment. This is an essential aspect of IMRT which allows for tighter margins by assessing and correcting for pancreatic motion due to breathing and variable GI filling and motility. IMRT allows for a reduction in morbidity as well as for dose escalation, and is the standard technique for definitive or neoadjuvant irradiation for PC.

The great advantage of IMRT is to produce a greater conformity of the dose distribution than with 3DCRT. This enables dose manipulation to create a sharp dose

fall off near the boundaries of tumor vs. critical normal organs. This may allow for a higher dose to be delivered to the tumor and a lower dose to the organs at risk of radiation injury or both. IMRT has proven to lead to less acute and late toxicity in multiple series [34, 35, 36]. Additionally, this enables excellent target volume coverage, and if needed, dose escalation to critical portions of the tumor near adjacent blood vessels. Neoadjuvant chemoradiation using an IMRT technique can facilitate a margin negative resection with customized treatment of high-risk volumes to maximize local control while at the same time minimizing dose to adjacent organs at risk [26].

Defining Treatment Volumes and Treatment Doses

One of the challenges of using IMRT is accurately defining the tumor or target volume. Appropriate treatment delivery is critically dependent on accurate target identification. IMRT was initially piloted in the postoperative setting and there was concern that use of IMRT could lead to an increase in local failures if the target volume was not accurately defined during planning and accurately treated daily. IMRT, in addition to image guided radiation therapy, has subsequently been used in the EORTC/US Intergroup/RTOG 0848 adjuvant trial after a successful pilot demonstrated no increase in local recurrence [37, 38]. A consensus postoperative atlas was created to help insure consistency in contouring (RTOG Consensus Panel Contouring Atlas for the Delineation of the Clinical Target Volume in the Postoperative Treatment of Pancreatic Cancer (https://www.rtog.org)). A modification of this atlas, based on targeting the most common sites of recurrence, has been published by Dholakia et al. [24] There has been even more debate as to the definition of target volume in the preoperative setting.

At MCW, in the neoadjuvant setting, the entire pancreatic head or body or tail are targeted, rather than just the visible gross tumor volume, along with the celiac axis and superior mesenteric artery and vein [26] (Fig. 1). This targets perineural spread of the tumor as well as microscopic lymph nodes adjacent to the large vessels coming off the aorta. Lesions that are near the portal vein, portal venous confluence, IVC or aorta, or the branches of the celiac artery (common hepatic artery) are also selectively targeted, if close to or involved by the primary lesion. Only suspicious nodes are targeted rather than comprehensively treating all nodal regions at risk. There is even more debate about targeting unresectable disease as this is often the setting where selective dose escalation is needed. CT often underestimates target volume whereas MRI may offer better soft tissue resolution and more accurate target definition. An international consensus document outlining MR-based delineation is now available [39].

Achieving a negative margin resection in the setting of tumor abutment or invasion of adjacent vessels is particularly challenging. Margin negative resection offers the best chance of cure. The presence of positive margins leads to inferior survival and increased local recurrence. Wang et al. used IMRT to not only treat the

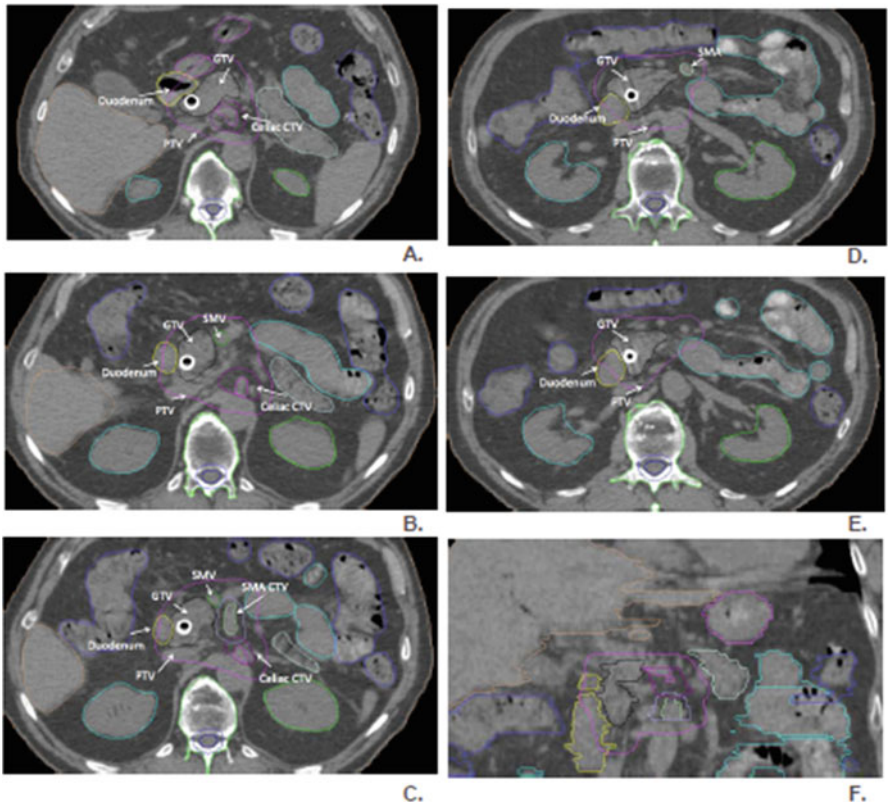

Fig. 1 Representative target volumes for preoperative treatment of resectable adenocarcinoma of the pancreatic head; (**a**–**e**) axial slices from superior to inferior, (**f**) coronal view. *GTV* gross tumor volume, *SMV* superior mesenteric vein, *SMA CTV* superior mesenteric artery clinical target volume, *CTV* celiac artery clinical target volume, *PTV* planning target volume

pancreas and adjacent vessels, but to use a higher dose (56 Gy) near the tumor vessel interface while treating the rest of the irradiated volume with a lower dose per fraction (50.4 Gy). Dose painting or simultaneous integrated boost technique is possible with IMRT, and this has resulted in a statistical trend towards increased resection in patients who received this boost without an increase in toxicity [40]. A similar approach was taken by Huang et al. with delivery of higher doses (56 Gy) to areas of vessel invasion using a combination of PET and CT for tumor definition and lower doses (50.4 Gy) to subclinical disease. In the 23 of 25 patients with borderline resectable disease who went on to resection, 22 (96%) had negative margins (>1 mm) [41]. Dose escalation is challenging as the normal tissues adjacent to the pancreas are very dose sensitive. IMRT along with daily image guidance can allow for delivery of higher doses than 3D conformal plans with better dose sparing of the adjacent

stomach, duodenum, bowel, and kidneys. The dose can be escalated to pivotal portions of the tumor, such as the retroperitoneal margin or tumor further away from the GI tract, while pulling dose away from the adjacent normal organs [42]. Even some patients with locally advanced PC can receive neoadjuvant dose escalated radiation and go on to margin negative resections in this setting [42–44]. IMRT as a definitive therapy has been reported in a number of studies [34, 38, 42, 43].

Altered Fractionation Schemes for Pancreatic Cancer

Historically, radiation therapy for PC, such as IMRT, has typically applied a conventional or "fractionated" treatment course; the radiation therapy is typically broken up over approximately 4–6 weeks of daily treatments. This delivery approach was used for a variety of reasons. First, radiation therapy was given using a two-dimensional treatment technique, which made the ability to visualize normal structures (such as the small bowel, large bowel, or stomach) extremely difficult. Therefore, doses of radiation therapy had to be given in a manner that was within the dose tolerance of these normal structures. Thus, very few options were available to give selectively higher doses to a tumor and spare normal structures, and most patients with PC were treated with low doses of radiation therapy (45–54 Gy) given over 25–28 fractions. This dose selection was largely controlled by the radiation tolerance of the normal tissues near the pancreatic tumor (small bowel and stomach). Technological changes in the ability to deliver radiation therapy over the past 15 years have presented a considerable opportunity to alter the way radiation therapy is delivered and has resulted in the ability to deposit high doses of radiation therapy to a tumor over a shorter treatment time. Considerable investigation has taken place over the past 10 years examining the use of higher doses of radiation therapy with shorter treatment schedules (Fig. 2).

A commonly used modality for the treatment of patients with PC is stereotactic body radiation therapy (SBRT) [45]. SBRT is a rapidly growing radiation therapy technique with applications in numerous malignancies [45]. This is an especially exciting area of radiation therapy delivery that results in a different mechanism of cell kill than conventionally fractionated radiation therapy. Treatments with SBRT are typically given over five or fewer fractions. SBRT has been extensively studied in PC with reports having been published from numerous, single institution series [46–55]. There are several conceptual advantages to the use of SBRT in patients with PC. First, the treatment course with SBRT typically takes less than 2 weeks, which is considerably shorter than conventionally fractionated radiation therapy, which extends over a time of 5–6 weeks. This shorter treatment course may permit an earlier return to systemic therapy. The mechanism of cell kill in SBRT may also hold advantages over conventionally fractionated radiation therapy, particularly for PC. For example, SBRT provides an ablative mechanism of cell kill as compared with conventional therapy [45]. However, SBRT for PC may also carry risks. A

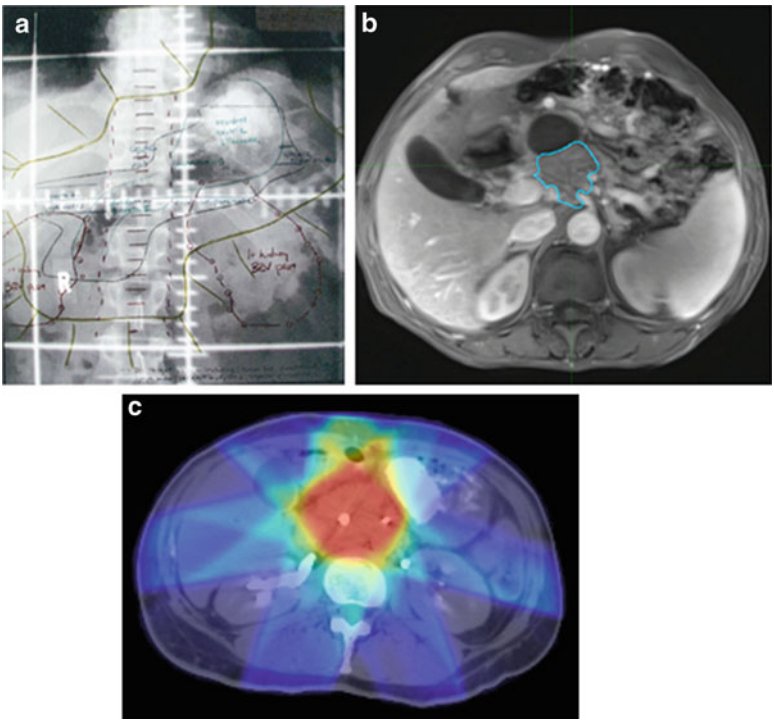

Fig. 2 (**a**) Represents a historic radiation treatment plan using 2D planning techniques, little ability to visualize normal structures or adapt dose accordingly. (**b**) Reflects modern era tumor contouring using MRI. (**c**) Radiation dose deposition (red represents high/prescription dose, blue represents lower radiation dose). Structures such as the small bowel and kidney can be seen clearly and avoided

higher radiation dose per fraction in close vicinity to the small bowel and stomach carries a risk of late toxicity to these organs. In addition, the treatment volumes with SBRT are usually smaller than conventionally fractionated radiation therapy. This may lead to a theoretically higher risk of marginal miss and regional nodal recurrence. While there are numerous theoretical advantages to the use of SBRT, there is a near complete absence of randomized data that has compared SBRT to conventionally fractionated radiation therapy. Table 2 summarizes the current SBRT series that have been published in patients with locally advanced PC. In addition to the multiple series that have described the use of SBRT for PC, recent publications have also examined the use of different fractionation schedules [42]. Krishna et al. present a range of doses and fractionation schedules, most of which are much shorter than fractionated treatment schedules ranging from 5 fractions to 28 fractions. Several patients were treated with fractionation schedules between 10 and 15 fractions. These would not typically be considered SBRT schedules, however these treatment courses do represent a different approach as compared with more conventional and

Table 2 Select series of SBRT in locally advanced pancreatic adenocarcinoma

Author	Year	Dose of radiation	Number of patients	1 Year freedom from local progression	Overall survival months	Acute/late grade 3 or higher toxicity
Polistina [54]	2010	30 Gy/3	23	50%	10.6	0%
Schellenberg [56]	2011	25 Gy × 1	20	94%	11.8	15%/20%
Lominska [57]	2012	20–30 Gy/3–5	28	86%	5.9	4%/7%
Gurka [55]	2013	25 Gy × 1	10	40%	12.2	0%/0%
Chuong [49]	2013	20–50 Gy/5	16	81%	15.0	0%/5.3%
Herman [47]	2015	33 Gy/5	49	78%	13.9	12.2%/10.6%
Koong [34]	2004	25 Gy × 1	6	100%	8.0	33%
Hoyer [50]	2005	15 Gy × 3	22	57%	5.4	79%/94%
Koong [58]	2005	25 Gy × 1	16	94%	8.25	12.5%
Schellenberg [51]	2008	25 Gy × 1	16	100%	11.4	19%/47%
Chang [59]	2009	25 Gy × 1	77	95%	11.9	5%/13%
Mahadevan [53]	2010	24–36 Gy/3	36	78%	14.3	41%/6%

prolonged treatment courses. Such fractionation approaches may have considerable advantages when compared with more prolonged, fractionated treatment courses. A comparison of these treatment approaches to SBRT approaches may be prudent for future study.

Technological advances in radiation therapy have enabled dramatic changes in dose and fractionation schedule for patients with PC. There is an obvious need for a randomized clinical trial comparing different fractionation schedules in patients with localized PC to determine how such treatments effect pattern of failure and patient survival.

Results from the Medical College of Wisconsin

Between 2009 and 2016, 245 consecutive patients completed neoadjuvant therapy and surgery at MCW for biopsy-proven PC. Of the 245 patients, 126 (51%) had resectable PC and 119 (49%) had borderline resectable PC; the median age at the time of cancer diagnosis was 65 years (interquartile range [IQR]: 12). Neoadjuvant therapy for the 245 patients consisted of chemotherapy alone in 38 (15%), chemoradiation in 83 (34%) or both in 124 (51%) patients. Of the 126 patients with resectable PC, 90 (71%) received chemoradiation, including 11 (9%) patients who were treated with induction chemotherapy prior to chemoradiation. The remaining 36 (29%) patients with resectable tumors were treated with chemotherapy alone. The preferred neoadjuvant treatment regimen for patients with borderline resectable PC consisted of 2 months of induction chemotherapy followed by

chemoradiation, and 113 (95%) of the 116 patients with borderline resectable PC were treated with both therapies. Overall, 207 (85%) of the 245 patients received chemoradiation which was gemcitabine-based chemoradiation in 164 (79%) and capecitabine-based chemoradiation in 43 (21%). Of the 162 patients who received chemotherapy, 77 (48%) patients received FOLFIRINOX, 79 (48%) received combination chemotherapy with either a 5-fluorouracil or a gemcitabine backbone, and 6 (4%) patients received gemcitabine monotherapy.

Of the 245 total patients, 192 (78%) underwent a standard pancreaticoduodenectomy (PD), 30 (12%) underwent a distal pancreatectomy, 17 (7%) underwent a total pancreatectomy and 6 (3%) underwent a pylorus-preserving PD. Vascular reconstructions were performed in 73 (30%) of the 245 patients due to tumor encasement. Margin negative (R0) resections were achieved in 219 (89%) patients and 147 (60%) had lymph node negative (N0) disease. Interestingly, of the 38 patients who did not receive neoadjuvant chemoradiation, only 16 (42%) had N0 disease and 22 (58%) had N1 disease. The majority of tumors were T3 ($n = 154$, 63%) and there was no difference in T stage with or without chemoradiation. However, a complete tumor response (T0) was observed in 6 (2%) patients and all 6 patients had received neoadjuvant chemoradiation. Data regarding PNI was included in the pathology report of 239 patients, and 152 (64%) had PNI and 87 (36%) patients did not. PNI was observed in 31 (82%) of the 38 patients who did not receive neoadjuvant chemoradiation as compared to 121 (58%) of the 207 patients who received neoadjuvant chemoradiation ($p = 0.01$).

Additional adjuvant therapy was administered to 144 (59%) of the 245 patients, and the remaining 101 (41%) patients were observed. Of the 144 patients who received postoperative therapy, adjuvant therapy consisted of chemotherapy in 116 (81%), chemoradiation in 3 (2%), and both in 25 (17%). All 245 patients underwent routine surveillance at 3–4 month intervals with physical examination, laboratory studies, and CT imaging. At a median of 25 months, 136 (55%) of 245 patients developed recurrent disease. Recurrent disease was assessed radiographically and rare cases were confirmed with a tissue biopsy. The site(s) of first disease recurrence were classified as local (peripancreatic or perivascular recurrences; Fig. 3), regional (peritoneal or abdominal wall recurrences; Fig. 4), distant (all other recurrence sites), or multisite.

For all 245 patients, disease recurrence was local in 19 (8%), regional in 17 (7%), distant only in 76 (31%) patients, and multisite in 24 (10%) patients. The median time to recurrence from the date of diagnosis for patients with local, regional, distant, and multisite recurrences was 18.4 months, 11.7 months, 15 months, and 15.1 months, respectively. Of the 100 patients with distant recurrences, the liver was the most common site of recurrence. The median overall survival was 36.5 months for all 245 patients. The median overall survival by first site of recurrence for patients with no recurrence, local, regional, distant, and multisite recurrence was: not reached; 31.5 months; 21.4 months; 24.8 months; and 20.6, respectively.

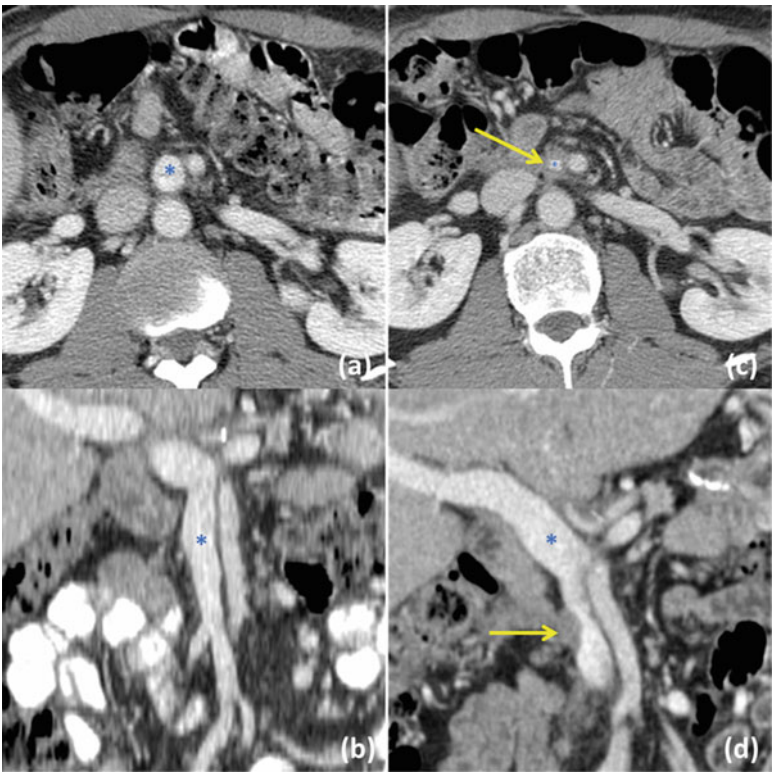

Fig. 3 Local tumor recurrence. Immediate postoperative (**a**,**b**) and 1-year follow-up (**c**,**d**) CT portal-venous phase images in the axial and coronal planes. Note the normal diameter of the SMV (blue *) and the normal tissues surrounding the SMV on immediate postoperative exam. 1-year follow-up exam after surgery demonstrates locoregional tumor recurrence encasing the SMV (blue *) for 360° (yellow arrow)

Conclusion

PC spreads quickly to local-regional perineurium due to the rich innervation of the pancreas by the autonomic nervous system. If patients live long enough, they will be susceptible to local recurrence in the neural tissue enveloping the celiac artery, SMA, or hepatic artery; such local recurrences are anatomically reproducible and a very consistent form of disease recurrence. Isolated local failure is uncommon when median survivals are short – as median survival increases, local recurrences may become more common. Isolated tumor cells in perineural tissue may experience a privileged environment and be less effectively treated with systemic therapy than, for example, microscopic metastatic disease in liver or lung. Such may not be the

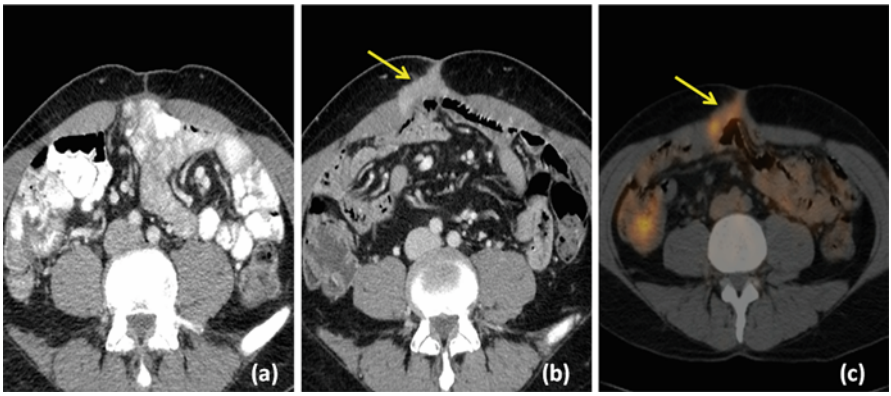

Fig. 4 Regional tumor recurrence in the periumbilical abdominal wall. Short-term postoperative CT exam (**a**) and 2-year follow-up postoperative CT (**b**) and PET/CT (**c**) exams at the same level. Note the new mass (yellow arrow) in the periumbilical abdominal wall region overlying the small bowel loops which is shown to be PET FDG positive (yellow arrow)

case if perineural tumor infiltration is treated with chemoradiation, especially when given preoperatively prior to the immune suppressive effects of surgery and the hypoxic tissue disruption that occurs following pancreatectomy. There is tremendous excitement over the emergence of altered fractionation schemes, techniques for tumor targeting using real-time MRI, and dose/schedule innovations which may make the delivery of neoadjuvant radiation easier and less toxic. In the opinion of the authors, the failure to incorporate modern radiation therapy techniques into the treatment schemas of patients with operable PC could be an error and one that will become noticeable when survival durations increase due to more effective systemic therapies.

Cross-References

- ▶ Arterial Resection in Pancreatic Cancer
- ▶ Borderline Resectable Pancreatic Cancer
- ▶ Neoadjuvant Chemotherapy in Pancreatic Cancer
- ▶ Role of Radiotherapy in Locally Advanced Pancreatic Cancer

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Part III

New Directions

Development of Novel Diagnostic Pancreatic Tumor Biomarkers

Lucy Oldfield, Rohith Rao, Lawrence N. Barrera, and Eithne Costello

Contents

Abstract

As the incidence of pancreatic ductal adenocarcinoma cancer (PDAC) increases, the need to improve the outcome for patients with this deadly disease becomes all the more pressing. Earlier detection of PDAC has the potential to improve

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survival, and biomarkers that enable earlier diagnosis are sought after. Some of the challenges associated with developing new diagnostic biomarkers for PDAC are reviewed here, including the need for appropriate control groups and the necessity to account for established confounding factors such as obstructive jaundice. High-risk groups, including individuals with new-onset diabetes, are discussed, and the findings of studies utilizing samples from pre-diagnostic cohorts to monitor changes in biomarker levels occurring in the weeks and months prior to diagnosis of PDAC are appraised. Progress toward identification of specific biomarker types is provided, and a variety of sources of biomarkers are examined, including blood, urine, pancreatic juice, gut lavage fluid, and extracellular vesicles. Additionally, a range of biomarker types are reviewed, including protein biomarkers, circulating tumor cells, circulating tumor DNA, and microRNAs. New developments with respect to emerging biomarkers, such as metabolites, are also examined. While progress to date has been slow, clear advances are being made, and the promise of biomarkers with clinical utility is in reach.

Keywords
Pancreatic cancer · Biomarkers · New-onset diabetes · Obstructive jaundice · Early detection

Introduction

The majority of cancers of the pancreas are histologically classified as pancreatic ductal adenocarcinoma (PDAC). For 80% of patients, the diagnosis of PDAC comes after the disease has spread locally or to the liver and other organs. This excludes surgery and severely limits curative treatment options. The overall 5-year survival of 3–5% for pancreatic cancer patients has not improved for many decades and is attributed at least in part to diagnosis occurring at a time when medical intervention does not significantly alter the outcome. PDAC is no longer considered to be a symptomless disease. However, nonspecific symptoms such as backache or lethargy have many possible underlying causes, and pancreatic cancer remains a very challenging disease to detect in the early stages. Overt or alarming symptoms, such as obstructive jaundice, often manifest late in the course of the disease. Almost half of pancreatic cancer patients are diagnosed following an emergency presentation to hospital.

At What Point in PDAC Disease Progression would Biomarker-Facilitated Detection Lead to an Improvement in Patient Outcome?

The World Health Organization (WHO) advocates that for 30% of all cancers, an early diagnosis determines whether the patient can be cured (www.who.int/cancer/en/index.html). Certainly, in the case of pancreatic cancer, patients eligible for

potentially curative surgery have a better prognosis than those with locally advanced or metastatic disease who are not amenable to surgery. Thus, biomarkers that increase the proportion of patients with tumors that are resectable could significantly enhance the overall survival [1].

The term biomarker has been defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [2]. Biomarkers that can facilitate earlier detection of pancreatic cancer are sought after, and much investment has taken place over several decades. However, despite a large number of publications, CA19-9, an epitope of sialylated Lewis blood group antigen, remains the sole biomarker that is in routine use for managing patients with PDAC [3, 4]. A number of factors underpin the failure to translate candidate biomarkers into clinical use for pancreatic cancer diagnosis. PDAC, ranked 11th in terms of incidence, is not as common as other cancers. Moreover, as the vast majority of patients are ineligible for surgery, the availability of pancreatic cancer tissue has in the past been limiting. Until recently, tissue samples were not available at all from PDAC patients who did not undergo surgery. Currently, most patients are diagnosed by fine-needle aspiration (FNA), which yields only small quantities of material for research purposes. PDAC tissue is composed of several cell types, which can potentially complicate biomarker studies using tissue. Moreover, it is now understood that PDAC tumors are characterized by high levels of genomic instability and heterogeneity [5, 6], which may alter the pattern of some biomarkers from patient to patient.

Understanding the nuances of PDAC is critical to the study of diagnostic biomarkers. Nowadays it is uncommon to see a PDAC biomarker study that does not include samples from patients with chronic pancreatitis as controls, alongside healthy controls. However, important additional controls are often sadly lacking. A majority of PDAC patients have tumors involving the pancreatic head, which is associated with obstructive jaundice [7]. Jaundice leads to a buildup of proteins in the circulation and can give rise to false-positive findings in blood-borne biomarker studies [8–10], so should be accounted for. It may also be important to consider other comorbidities, such as diabetes, which are discussed later.

Finally, the aim of diagnostic biomarker studies is to discover biomarkers that will allow disease detection at a time when therapeutic intervention is feasible and will improve prognosis. With current treatments, facilitating the detection of PDAC that is already metastatic is unlikely to provide any benefit to patients, and earlier intervention is necessary. Surgery combined with chemotherapy currently provides the only chance of pancreatic cancer cure. Thus, detecting PDAC when it is still amenable to potentially curative surgical resection or when chemotherapeutic intervention would enable surgery by causing downstaging of locally unresectable disease could improve overall survival. The most recent European Study Group for Pancreatic Cancer trial, ESPAC-4, demonstrated that the adjuvant use of gemcitabine plus capecitabine gave a 5-year survival rate approaching 30%. However, the search for biomarkers that will inform the presence of PDAC that is resectable is hampered by the fact that most patients are diagnosed when the disease is advanced, and the samples provided for research by such patients may not provide

information on the characteristic of early-stage disease. Interrogating samples obtained in months prior to PDAC diagnosis could potentially provide insight into biomarkers that appear earlier in the timeline of PDAC disease progression. The use of cohort studies, such as the European Prospective Investigation into Cancer and Nutrition (EPIC) [11] or UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS) [12, 13], has begun to provide insight into markers that are potentially indicative of early disease, as well as markers that are not [14–16]. Recent evidence that preneoplastic lesions are capable of disseminating into the bloodstream [17] begs the question of whether biomarkers of such lesions are required, in order to be sure of detecting early disease. Progress toward such biomarkers is discussed later.

The Current Gold Standard

The best application of CA19-9 is in predicting clinical course during and following treatment, with a rise in CA19-9 levels potentially signifying disease recurrence. CA19-9 has a sensitivity of approximately 80% for PDAC diagnosis [18]. Around 5% of people are Lewis ab negative and as a consequence do not secrete CA19-9 [19]. The specificity of CA19-9 for PDAC diagnosis is also around 80% [18]. This relatively low specificity is due to the fact that CA19-9 is elevated in benign conditions, such as pancreatic inflammation [3], and precludes the use of CA19-9 in large-scale population screening, because of the large number of false positives that would be generated. The values for sensitivity and specificity quoted above were attained by testing CA19-9 in individuals already diagnosed with PDAC. Recent studies have however attempted, using pre-diagnostic cohorts, to evaluate whether CA19-9 levels increase prior to clinical presentation of PDAC. Using case-control samples gathered as part of the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS), CA19-9 levels >37 U/mL were found, at a specificity of 95%, to have a sensitivity of 68% up to 12 months prior to diagnosis. At the same specificity, sensitivity decreased to 53% up to 24 months prior to diagnosis. An independent study found CA19-9 to have much lower sensitivity for PDAC detection pre-clinically [20]. In pre-diagnostic sera obtained from cases of pancreatic cancer enrolled in the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO), the sensitivity of CA19-9 for PDAC was 17.2% at 95% specificity for patients 1–12 months from diagnosis [20]. Enhancing the performance of CA19-9 for pancreatic cancer diagnosis by adding additional biomarkers or early indications/symptoms of PDAC is clearly desirable [21–23].

Carcinoembryonic antigen (CEA) has been used with some accuracy in the diagnosis of pancreatic cancer and is currently used clinically alongside CA19-9 and imaging. CEA is overexpressed in other tumors, such as colorectal tumors [24], and as such lacks specificity as a stand-alone marker for pancreatic cancer. A recent meta-analysis of CEA as a diagnostic tool found that for identification of pancreatic cancer the mean sensitivity was 44% (95% CI 38.5–50.0%) and the mean specificity was 87% (95% CI 82.5–91.2%) [25]. In comparison to CA19-9, the relatively poor sensitivity indicates that CEA is inferior in identifying PDAC. However, the similar

specificity of CEA when compared to CA19-9 highlights its utility in correctly identifying subjects who do not have PDAC.

Alternatively, using CA19-9 or other biomarkers in combination with early indications/symptoms of PDAC may prove useful. In a study of more than 11 million electronic patient records, from 562 general practitioner practices in the UK, in which 2773 patients with PDAC were diagnosed and compared with over 15,000 controls, it was found that patients with PDAC made a median of 18 visits to their general practitioner in the year prior to diagnosis. Moreover, PDAC was associated with 11 alarm symptoms, including back pain, lethargy, and new-onset diabetes mellitus [26]. Understanding the patterns of early PDAC symptoms will provide the opportunity to combine these with available biomarker tests and lead to earlier PDAC diagnosis.

Considerations Regarding the use of Diagnostic Biomarkers

The sensitivity and the specificity required from a biomarker or biomarker panel depend largely on the intended use of that biomarker. Despite the high morbidity and mortality associated with pancreatic cancer, it is nonetheless a relatively uncommon disease. The current lifetime risk of being diagnosed with pancreatic cancer is 1 in 71, although the incidence of pancreatic cancer is expected to rise significantly in the next decades. This relatively low overall lifetime risk of developing pancreatic cancer argues against population screening, particularly in the absence of highly sensitive and specific biomarkers. Population screening also relies on there being effective treatments, and since surgery is currently the only treatment that guarantees cure, biomarkers would have to enable the detection of resectable disease or disease that could be downstaged to enable resection. Finally, for biomarker tests to be widely used, they should be safe, inexpensive, and acceptable to patients. There are currently no biomarkers that fulfill the criteria for general population screening for pancreatic cancer. Since the incidence of PDAC is higher in groups at high risk of developing the disease, such groups are attractive for the testing of new candidate biomarkers. Moreover, new biomarkers that can further stratify for risk within high-risk groups are greatly sought after.

High-Risk Groups

Individuals with an Inherited Risk of Pancreatic Cancer

Approximately 10% of patients with PDAC have a family history of the disease, and a proportion of these families have a pattern of risk consistent with autosomal dominant predisposition [27]. For this subset of families, screening is justified with a view to earlier disease detection. In this book, the chapter entitled “Secondary Screening for Inherited Pancreatic Cancer” describes both the biomarkers currently available and the approaches taken for screening risk populations. By contrast, the

great majority (over 90%) of PDAC cases cannot be predicted on the basis of family history and are referred to as sporadic. No current screening modality is available for sporadic pancreatic cancer. As such, diagnosing sporadic pancreatic cancer at a curable stage is currently a huge unmet need.

New-Onset Diabetes

The relationship between PDAC and diabetes mellitus (DM) is complex. Long-standing DM increases the risk of PDAC by approximately twofold [28]. However, it is now evident that PDAC causes DM [28]. Approximately 40–80% of PDAC patients have DM or glucose intolerance at the time of diagnosis of cancer [29, 30], although it often goes undiagnosed. By following individuals newly diagnosed with type 2 DM, it became apparent that 1 in 100 patients is diagnosed with PDAC within 3 years of the diagnosis of DM, representing a significantly elevated risk (between five- and eightfold depending on the age of the individual) of PDAC compared to individuals without a new diagnosis of DM [30]. Further analysis suggested that these individuals had early-stage PDAC at the time they are diagnosed with DM. In effect, diabetes was secondary to PDAC and as such is referred to as type 3C diabetes and could be an early warning sign of the presence of cancer. The average time between the diagnosis of DM and the subsequent diagnosis of PDAC is 13 months [28]. This provides a significant window for earlier detection of PDAC and is especially significant because of the high proportion of PDAC patients (>50%) affected by new-onset DM prior to cancer diagnosis. It makes new-onset DM the largest high-risk group for pancreatic cancer.

However, the incidence of diabetes in the general population is rising, and understanding the various subtypes is critical. It is unfeasible, with current modalities, to screen all individuals newly diagnosed with diabetes for PDAC. Undoubtedly, screening this high-risk population would be facilitated if diagnostic biomarkers were available that could enrich for those individuals with new-onset DM who are most likely to have PDAC (making additional screening of this much smaller group feasible), and progress has been made. Plasma levels of adrenomedullin were found to be higher in PDAC patients with diabetes compared to PDAC patients without diabetes and were significantly higher in PDAC patients with diabetes compared to non-cancer subjects with diabetes [31]. The sensitivity and specificity of adrenomedullin (as a single marker) in distinguishing PDAC cases from non-PDAC controls were 69% and 81%, respectively [31]. Pancreatic polypeptide (PP), a hormone secreted by islet cells, has been evaluated for its ability to distinguish pancreatic cancer-associated diabetes from type 2 diabetes [32]. The serum OPG levels of 18 subjects with new-onset diabetes, half of whom had pancreatic cancer- associated diabetes, were evaluated at time intervals following a mixed meal. Serum PP levels were lower in the pancreatic cancer patients at 30 min following a mixed meal. Differences were noted between patients with a tumor in the head compared to the tail of the pancreas [32].

Using gene array analysis, Huang et al. [33] identified vanin-1, a pantetheinase found on the extracellular membrane of epithelial and myeloid cells, as upregulated in peripheral blood samples from patients with PDAC and DM, compared with PDAC patients without DM and control individuals with longstanding DM and healthy controls.

Fully understanding and exploiting the knowledge that individuals with new-onset DM are a high-risk group for PDAC could make a significant impact on the survival of PDAC patients, potentially enabling detection of the disease when it is at a treatable stage.

Biomarkers for Precursor Lesions

Pancreatic intraepithelial neoplasia (PanIN) lesions, intraductal papillary mucinous neoplasms (IPMN), and mucinous cystic neoplasms (MCN) are precursor lesions for sporadic PDAC. These lesions have been well defined in recent years with global consensus guidelines published regarding their management [34, 35]. Cystic precursor lesions are usually discovered as incidental findings on radiological imaging as they are asymptomatic. Given the high mortality associated with pancreatic cancer, diagnosis of these precursor lesion assumes high importance if we are to improve outcomes. Systems capable of accurately predicting malignant transformation of these lesions are hugely sought after.

Technological advances in cross-sectional imaging have improved the ability to detect abnormalities of the pancreas. As a consequence, an increase in the diagnosis of cystic neoplasms of the pancreas has occurred, with an estimated 13% of the population currently diagnosed with incidental cystic pancreatic lesions during cross-sectional imaging [36]. Currently there is no validated serum biomarker accurately able to predict malignant transformation of these lesions, and we are dependent on serial radiological surveillance or invasive endoscopic procedures to characterize them. To compound the problem, PanIN lesions lack specific symptoms for clinical diagnosis and are too small to be easily characterized with current imaging modalities [37]. There has been a global impetus to develop a biomarker panel able to facilitate accurate diagnosis of localized PDAC and neoplastic lesions, which would translate to early diagnosis, curative resection of localized tumors, and ultimately improved survival.

Circulating epithelial cells (CECs) have been reported in preinvasive and early tumorigenesis stages in mouse models [38]. Pancreatic epithelial cells from mice with PanIN lesions, but devoid of tumors, were shown to have acquired invasive properties and were detected in peripheral blood. Interestingly, these circulating cells had undergone epithelial-to-mesenchymal transformation, a process characterized by the loss of epithelial features and the gain of mesenchymal characteristics, such as, invasiveness and resistance to apoptosis.

Detection of circulating epithelial cells via a venous sampling test to diagnose early cancer holds great appeal. Rhim et al. [39] undertook a prospective study aimed at detecting circulating epithelial cells (CEC) of pancreatic origin. Forty-eight

patients were recruited from three groups – healthy subjects, individuals with cystic neoplasms of the pancreas not warranting surgery, and patients with PDAC. High counts of CEC were detected in patients with PDAC (7/9), but interestingly, 40% of individuals with noninvasive pancreatic lesions demonstrated CEC in their circulation. This subgroup of patients had no high-risk stigmata predisposing to development of PDAC. Although it was not known if all patients where CEC were detected went on to develop tumors, understanding the significance of the presence of CEC will be important for their utilization as a biomarker in the future.

There is much current interest in the exploitation of microRNAs (miRNAs) as markers that could potentially predict the malignant transformation of precursor lesions. miRNAs are noncoding RNAs containing 18–24 nucleotides that negatively regulate gene expression. They are described in greater detail in a later section of this chapter; however, their role in the detection of precursor lesions is dealt with here. Habbe et al. [40] undertook profiling of miRNA in the tissue of IPMN lesions that had been surgically resected. The study focused on two miRNAs, miR-21 and miR-155, for their role in identifying IPMN undergoing malignant transformation. Sixty-four samples were analyzed, including low-grade dysplasia (n = 13), moderate dysplasia (n = 31), and high-grade dysplasia (n = 20), with both miRNA-21 and miRNA-155 found to be overexpressed in tissue from IPMN compared to normal pancreatic tissue. Significant upregulation of both miRNAs was observed in patients with IPMN associated with high-grade dysplasia compared to those with low-grade dysplasia. miRNA-155 was overexpressed in patients with intestinal or pancreatobiliary histological subtype of IPMN which have an increased tendency for malignant transformation. Further profiling of these miRNAs in pancreatic juice showed that miRNA-155 was elevated in 60% of IPMN samples while barely detectable in subjects with benign pancreatic conditions such as chronic pancreatitis, indicating that miRNA-155 could serve as a biomarker for IPMN in pancreatic juice analysis.

A subsequent multicenter retrospective study analyzed miR-21, miR-155, and an additional miRNA, miR-101, from laser-microdissected invasive (n = 65) and noninvasive (n = 16) IPMNs, as well as normal pancreatic ductal tissues (n = 5) [41]. miR-21 and miR-155 were significantly overexpressed in invasive IPMN compared to noninvasive IPMN and normal tissues. By contrast, miR-101 was more highly expressed in noninvasive IPMN and normal tissues compared to invasive IPMN. Thus all three miRNAs were altered in expression between invasive and noninvasive IPMN and offer potential discrimination between these states [41].

Genome-wide profiling of miRNA provided additional insight into miRNAs that can distinguish between high- and low-risk IPMNs [42]. In a discovery phase, containing surgically resected IPMNs from 19 high-risk and nine low-risk cases, six miRNAs, miR-100, miR-99b, miR-99a, miR-342-3p, miR-126, and miR-130a, were identified as downregulated in the high-risk IPMNs compared to that low-risk group. The trend was observed also in the validation phase, which contained similar numbers of IPMNs. The above studies show the possible use of miRNAs as aids to clinical management in distinguishing IPMNs with malignant potential, and endoscopic ultrasound has facilitated the accurate sampling of IPMN. Nonetheless, less

invasive tests, ideally using fewer miRNAs, are desirable. Li and colleagues [43] measured 735 miRNAs in blood serum, selecting 18 miRNA for validation. Although a number of miRNAs were identified that could distinguish pancreatic cancer patients from healthy controls, miR-1290 was the best-performing miRNA. It was found to be significantly elevated in pancreatic cancer patients compared to healthy controls and also in patients with IPMN compared to healthy controls. Analysis of cancer tissue indicated higher expression of miR-1290 transcripts in both pancreatic cancer and IPMN tissue compared to normal pancreatic ducts.

Families of Biomarkers

Protein Biomarkers in Biological Fluids

Proteomic profiling of a variety of different biological samples has been undertaken with the aim of identifying sensitive and specific diagnostic biomarkers of PDAC. Each sample type offers unique advantages but also carries distinctive challenges.

Blood as a Source of Protein Biomarkers

By far the most common body fluid used in diagnostic protein biomarker studies for pancreatic cancer is blood in the form of serum or plasma. Although cheap and minimally invasive to obtain, blood contains proteins that are not specific to a particular organ of the body. Furthermore, some proteins in blood are present in very high abundance and may mask others which are present in trace amounts [44]. Nevertheless, a blood test that could enable earlier diagnosis of pancreatic cancer would represent a significant advance. Moreover, given the heterogeneity within the overall population, it is widely considered essential that a biomarker test would consist of a panel of two or more protein biomarkers.

Biomarkers that can demonstrably distinguish pancreatic cancer at an earlier stage than is currently possible are desired. In this respect a significant development has been the use of pre-diagnostic human cohort studies, alongside genetically engineered mouse models of PDAC, to provide vital insight into proteins that are changing in abundance in blood in the weeks and months prior to overt pancreatic cancer. Nolen et al. [20] profiled the levels of 67 proteins in pre-diagnostic sera from PDAC cases and controls registered in the Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial. This afforded the opportunity to assess the performance of biomarker panels that had previously performed well in distinguishing PDAC cancer cases from controls when PDAC samples were taken at or post-PDAC diagnosis [22]. Previous analysis of 83 proteins in 333 PDAC patients and 144 patients with benign pancreatic conditions yielded a panel of CA19-9, OPG, and OPN which demonstrated a very promising sensitivity of 82.4% for PDAC detection at a specificity of 95%, yielding an AUC of 0.935. [22]. However, when tested in pre-diagnostic samples [20], the same panel offered poor classification power, demonstrating a sensitivity of 34%, a specificity of 84.7%, and an AUC of 0.547. A number of other candidates, which had shown good discriminating power

when tested in samples taken at the time of diagnosis of PDAC, also fared badly at distinguishing PDAC cases from controls when assessed in pre-diagnostic samples [20]. Jenkinson et al. [45] used the UKCTOCS pre-diagnosis samples to assess the performance of promising candidate diagnostic biomarkers prior to clinical presentation of PDAC. The serum levels of two candidates, intercellular adhesion molecule-1 (ICAM-1) and tissue inhibitor of metalloproteinases-1 (TIMP-1), were evaluated. Despite previous reports that these proteins were elevated in patients diagnosed with PDAC, neither protein was elevated in samples taken 0–12 months prior to PDAC diagnosis compared to non-cancer control samples [45]. Importantly, the study found that both proteins were significantly elevated in patients with obstructive jaundice secondary to either PDAC or gallstones. It was concluded that the failure of previous studies to account for biliary obstruction may have led to false-positive results. The above studies [20, 45] point to the difficulties of extrapolating alterations occurring prior to diagnosis from data acquired at or after the time of diagnosis. Moreover, failure to account for jaundice creates false-positive diagnostic signals in blood samples and continues to lead to the publication of poor-quality studies.

In a separate study, serum samples from the UKCTOCS collection of PDAC cases up to 4 years prior to diagnosis were subjected to proteomic biomarker discovery analysis [15]. Two-dimensional liquid chromatography-tandem mass spectrometry (LC-MS/MS) with isobaric tags for relative and absolute quantification (iTRAQ) of discovery samples ($n = 160$) led to quantification of 225 proteins in serum at 95% confidence. Of these circulating levels of thrombospondin 1 (TSP-1) were found to be reduced prior to diagnosis of PDAC. Multiple reaction monitoring (MRM), an LC-MS/MS technique for accurate protein quantification, along with Western blotting, was then undertaken to validate TSP-1 levels in a total of 472 human samples. Significant decreases in serum TSP-1 levels were observed in PDAC patients compared to controls and in KPC mice when they had cancer. Moreover, circulating TSP-1 levels were found to be reduced in PDAC cases compared to time-matched controls up to 24 months prior to PDAC diagnosis. For samples taken between 0 and 24 months prior to PDAC diagnosis, TSP-1 achieved an AUC of 0.69, while for CA19-9 the discrimination between PDAC cases and controls yielded an AUC of 0.77. Combined, TSP-1 and CA19-9 performed significantly better (AUC of 0.85). Finally, reduced TSP-1 levels were more frequently observed in PDAC patients with diabetes. This work highlighted the potential impact of diabetes on the performance of blood-borne biomarkers for PDAC.

Mirus et al. [46] used an antibody microarray with over 4000 features to profile proteins in plasma samples from the genetically engineered KPC mouse model of PDAC. In order to profile plasma from animals with preinvasive and early invasive PDA, plasma samples were interrogated from mice at 6–8 weeks and midway through the lifespan of animals, respectively. A total of 54 proteins were altered in mice in the preinvasive category with 25 proteins altered in mice in the early invasive category compared to controls. This study was complemented by comparing the proteins present in pre-diagnostic plasma samples from women in the Women's

Health Initiative (WHI) who were subsequently diagnosed with PDAC with control samples from women in the study who did not receive a diagnosis of cancer. In total, 88 proteins were altered in level in pre-diagnostic plasma compared to controls. Based on the mouse and human data, three candidate markers, ERBB2, ESR1, and TNC, were included in a panel which was evaluated for its ability to distinguish pre-diagnostic cancer cases from controls. The panel achieved an AUC of 0.68, and the performance improved slightly when CA19-9 was included in the panel.

Urine as a Source of Protein Biomarkers

Ease of accessing samples is an important consideration, and sample types that are readily obtained in a noninvasive manner, such as urine, are attractive as they would likely be acceptable to patients and cheap to obtain. Radon et al. [47] compared the protein profile of urine samples from healthy controls and patients with chronic pancreatitis and pancreatic cancer using in-gel tryptic digestion followed by liquid chromatography-tandem mass spectrometry (GeLC-MS/MS) analysis. Three markers, LYVE-1, REG1A, and TFF1, were selected for validation using ELISA. As a panel, these three markers performed well in distinguishing pancreatic cancer patients ($n = 192$) from healthy controls ($n = 87$). Areas under the receiver operating characteristic (ROC) curves (AUCs) of 0.89 and 0.92 were achieved in training and validation sets, respectively. Moreover, the panel was able to distinguish early-stage pancreatic cancer patients from healthy controls achieving AUCs of >0.9 when comparing PDAC stage I–II ($n = 71$) with healthy urine specimens. Further work to validate this panel prospectively is ongoing.

Pancreatic Juice/Whole gut Lavage Fluid as a Source of Protein Biomarkers

Pancreatic juice is secreted from the pancreatic ductal system and therefore has close physical contact with the tumor. This makes it an attractive source of biomarkers, as it may contain cancer-specific or cancer-enriched proteins actively secreted from the tumor or released through tumor shedding or necrosis. Indeed, proteomic profiling has revealed pancreatic juice to be rich in potential protein biomarkers [48], and the tumor markers CEA and CA19-9 are present in pancreatic juice [49] but not at higher levels than in serum. Collecting pancreatic juice poses a number of challenges. It is not easy to collect; the process can be invasive and may cause severe pancreatitis. Alternatives to analyzing pancreatic juice are therefore desirable. A new strategy for studying pancreatic juice proteins has been proposed by Rocker et al. [50]. A comparison was made between the protein profiles of whole-gut lavage fluid (WGLF) obtained during routine colonoscopy and pancreatic juice collected during surgery. The application of LC-MS/MS to the analysis of proteins contained within these fluids revealed a considerable overlap, with 90% of 104 proteins in pancreatic juice also present in WGLF samples. Likewise, 67% of proteins present in WGLF were identified in pancreatic juice. The study suggests that WGLF could be a surrogate biofluid for pancreatic juice and would enable an assessment of the pancreas in patients undergoing routine colonoscopies.

Circulating Tumor Cells

Circulating tumor cells (CTCs) are shed by a primary tumor or metastasis into the vasculature or lymphatics that then travel in an individual's circulatory system. Tumor cells may be shed passively by the primary tumor to enter circulation or are subjected to a more active process involving epithelial-to-mesenchymal transition.

Technological advances have created opportunities for the detection of CTCs in liquid biopsies. While there is mounting evidence that CTCs have prognostic value and are useful as surrogate response markers for the management of patients posttreatment, their utility in diagnosis is increasingly explored. Detection of CTCs is challenging due to their low number. In peripheral blood of individuals with metastatic cancer, the number of CTCs is estimated at one per 10^5 to 10^7 mononuclear cells. Individuals with nonmetastatic cancer have fewer CTCs. In addition, viable tumor cells shed into circulation are sequestered by the reticuloendothelial systems of the liver and spleen compounding their detection.

Enrichment techniques have been applied to improve CTC detection in blood (Fig. 1). These techniques target either physical properties of CTCs or biological properties or a combination of both. Centrifugation of cells in an isotonic medium can be used to separate tumor cells from mononuclear cells, as tumor cells have a different buoyant density, and commercial kits such as LymphoPrep™, Ficoll-Hypaque™, and Oncoquick® are available. Filtration techniques take advantage of the fact that tumor cells are comparatively larger than white blood cells; the isolation by size of epithelial tumor (ISET) cells is an example of one such filtration technique. However, both density and size distributions of CTCs are now known to overlap with peripheral blood mononuclear cells, and additional physical properties that could minimize separation of CTCs from blood cells have been explored. Dielectrophoresis is the motion of cells in the direction of increasing electric field intensity and offers opportunities for separating cell types. Enrichment techniques also take advantage of differential expression of cell-surface protein markers. Positive selection of CTCs has involved the use of immunomagnetic separation based on epithelial marker expression, e.g., EpCam or anti-mesenchymal antibodies or both, while negative selection has involved the depletion of mononuclear cells using well-established cell-surface markers for these cells (Fig. 1). Systems enabling cells to be separated using magnetic beads include Dynabeads (Invitrogen) and the system known as CellSearch (Veridex), which depends on expression of the epithelial marker, EpCam. Techniques to detect CTCs in peripheral blood include immunological assays such as immunohistochemistry or immunofluorescence. This enables an evaluation of tumor cell morphology; however, it depends on marker expression, and reliable markers of CTCs remain elusive. Nucleic acid-based tests enable the detection of mutated genes, gene transcripts, or miRNAs. Functional assays allow for detection of secreted proteins or an evaluation of the tumourigenicity of recovered cells in immunocompromised mice.

To date, most of the studies of CTCs involving pancreatic cancer patients have been aimed at understanding which techniques are applicable and have related findings to prognostic data [51]. Of note, in a study of 12 distinct metastatic cancer

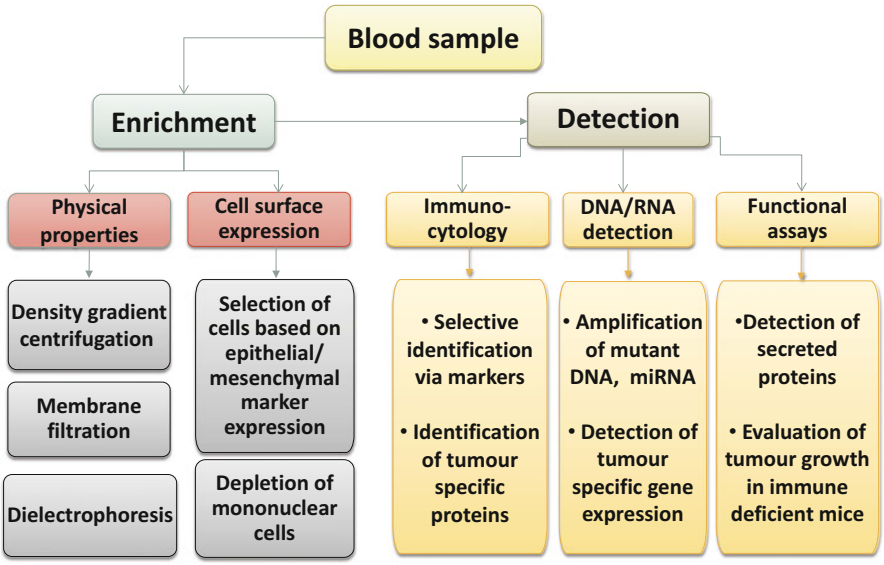

Fig. 1 Techniques employed for the enrichment and detection of circulating tumor cells in blood

types in which CellSearch was used to detect CTC, PDAC patients had the lowest levels of CTC [52]. This may reflect the limitation of using EpCam as a detection marker. When CellSearch was compared with ISET, a technique that relies on cell size, more CTCs were detected with ISET [53].

In terms of diagnosis, Ankeny et al. [54] recruited PDAC patients prior to treatment and employed a microfluidic CTC Chip (NanoVelcro) which targets EpCam. CTCs were detected in 54 of 72 patients with PDAC. In all cases, the KRAS mutations in CTC corresponded to those in the matching primary tumor. Using a cutoff value to >3 CTC in 4 mL of blood, CTC could identify patients with metastasis with sensitivity of 85.2% and specificity of 86.7%. This study demonstrates the potential of CTC as an aid to diagnosis of PDAC, and a larger validation study is in progress which will determine whether the cutoff established here is robust.

In summary CTC can be detected through the targeting of multiple cellular properties and indeed over 30 different techniques have been described. Research to date has provided evidence to support an association between CTC number and prognosis, although considerably more work is required before the potential of CTCs as an adjunct to PDAC diagnosis is exploited.

Circulating Tumor DNA

Circulating cell-free DNA (cfDNA) was reported nearly seven decades ago and continues to generate interest. The majority of circulating free DNA originates from

apoptosis or necrosis of cells, with white blood cells contributing to over 70% of the pool of circulating DNA. Tumor cells with their high mitotic rate undergo rapid apoptosis and release fragments of tumor-derived DNA into the circulation. Known as circulating tumor DNA (ctDNA), this subset of cell-free DNA can arise from apoptosis of primary tumor cells or lysis of CTCs. CfDNA has a low plasma half-life with rapid excretion through hepatic and renal metabolism [55], providing a small window for detection. ctDNA is present at a low level, but it has been identified with confidence using up to 5 mL of plasma [56]. Although ctDNA is readily identifiable in plasma fractions, dilution can occur, and diligence is required during analysis to prevent contamination with cellular DNA. Healthy individuals have an average of 30 ng/mL of cfDNA, but this becomes elevated sixfold in patients with solid tumors [57]. However, inflammatory states and benign tumors can also lead to elevated levels of cfDNA, and hence it lacks the necessary specificity as a stand-alone marker. Analyzing cfDNA for tumor-specific mutations helps to differentiate ctDNA from cfDNA. Combining ctDNA detection with analysis of tumor-specific mutations increases the sensitivity of ctDNA and has been shown to reflect disease stage and predict overall survival [57, 58]. Digital polymerase chain reaction (PCR) has shown the most promise among PCR-based techniques at identifying mutations, and studies have achieved 87–95% sensitivity and 99% specificity in detecting *KRAS* mutations in colorectal cancers [59]. Next-generation sequencing has been utilized in several studies to detect ctDNA and has the advantage of enabling analysis of multiple genes and the detection of novel mutations, making it highly specific.

Bettegowda and colleagues evaluated whether ctDNA was detectable using digital PCR in patients with various cancer types. ctDNA was detected in over 50% of PDAC patients with localized tumors and in over 80% of PDAC patients with metastatic tumors. Further studies including a meta-analysis have noted that the presence of ctDNA was associated with poor survival in PDAC [60]. The prognostic relevance of ctDNA carrying KRAS mutations in patients undergoing resection for PDAC was assessed. KRAS mutations were detectable in 31% of the cohort and were associated with poor survival. This possibly reflects the circulating micrometastatic burden [61].

In summary, ctDNA has been shown to have potential utility to predict survival in pancreatic cancer patients and may highlight patients liable to have early recurrence. However, refinements in detection will be required in order to use ctDNA as an aid for diagnosis.

MicroRNA

MicroRNAs (miRNA or miR) are a class of short, ~ 22 nucleotide, noncoding RNAs that regulate gene expression by binding to specific sites on the mRNA of protein-coding genes to direct their repression. These short single-stranded RNAs have been increasingly studied in recent years with more than 2500 human miRNAs registered to date (www.mirbase.org). miRNAs are now known to be important regulators of a variety of cellular processes, including development, differentiation, cellular

proliferation, and apoptosis. As regulators of multiple protein-coding genes, it is no surprise that dysregulation of miRNA can lead to the disruption of normal cell growth and development, resulting in a variety of disorders including cancer. miRNAs with regulatory roles in cancer have been studied extensively, with two distinct groups clearly categorized: those that are oncogenic and those whose depletion promotes tumorigenesis [62]. Tumor suppressor miRNAs are frequently downregulated in cancer. They inhibit the initiation and progression of pancreatic cancer by negatively regulating cell proliferation (miR-137 (63), miR-615-5p [64]), by facilitating apoptosis (miR-345 (65), miR-506 [66]), or through inhibition of cellular migration and invasion (miR-615-5p (64)). In contrast to tumor suppressor miRNA, oncogenic miRNAs (onco-miRNAs) are often found to be aberrantly overexpressed. Their upregulation has been shown to contribute to proliferation, migration, invasion, and inhibition of apoptosis. Upwards of 100 miRNA have been identified as being differentially expressed in pancreatic cancer [67]. A selection of recently reported miRNAs and their biological function in pancreatic cancer is summarized in Table 1.

Of those onco-miRNAs and tumor suppressor miRNAs found to play important roles in pancreatic cancer tumorigenesis and progression, several key candidates show potential as clinically viable stand-alone biomarkers, including miR-18a [68] miR-34a [69], miR-137 [63], and miR-1290 [70]. For example, miR-34a, a promoter of apoptosis and commonly deleted in human cancer, was recently identified from a number of miRNAs shown to exhibit p53-dependent upregulation upon DNA damage [69]. The significance of miR-34a in PDAC was demonstrated by the reduction or complete loss of expression of this miRNA in 11 pancreatic cancer cell lines. miR-34a has been measured in whole blood and sera, and its ability to distinguish pancreatic cancer from non-cancer controls has been highlighted in independent studies [71, 72], making it a promising candidate for early diagnosis of pancreatic cancer.

While there are an increasing number of studies revealing the potential of stand-alone markers as diagnostic tools, it is worth noting that single-miRNA biomarkers are frequently nonspecific. It is perhaps of greatest utility, therefore, to focus on comprehensive profiling of circulating miRNA and the creation of diagnostic panels. Several recent studies have addressed this need. Using microarray analysis coupled with RT-qPCR, Ganepola et al. [89] identified a panel of three circulating miRNA, miR-642b, miR-885-5p, and miR-22, differentially expressed in plasma from patients with PDAC compared to healthy controls and high-risk individuals. Validation of the combined targets demonstrated a high level of diagnostic accuracy for early-stage PDAC (sensitivity of 91%, specificity of 91%, and AUC of 0.97). Cote and colleagues found a panel of three miRNAs to be differentially expressed in plasma and bile from patients with PDAC compared to controls [67]. miR-10b, miR-155, and miR-106b displayed excellent accuracy in a validation cohort ($n = 120$) for distinguishing PDAC from chronic pancreatitis and normal pancreas (sensitivity and specificity were 95% and 100% in plasma and 96% and 100% in bile). In one of the largest discovery studies to date, 754 different miRNAs were examined in serum ($n = 205$) identifying 24 differentially expressed miRNAs in patients with

Table 1 Selected miRNAs with defined roles in pancreatic cancer, reported from 2014 to 2016

MiRNA	Role	Expression	Function in pancreatic cancer	Reference
miR-29a	Tumor suppressor	Down	Decreases cell proliferation and migration via inhibition of MUC1	[73]
miR-137	Tumor suppressor	Down	Inhibits cell proliferation	[63]
miR-192	Tumor suppressor	Down	Inhibits cell proliferation, viability and EMT via targeting of PAI-1	[74]
miR-200a	Tumor suppressor	Down	Inhibits EMT, cell migration, and invasion	[75]
miR-219-1-3p	Tumor suppressor	Down	Decreases proliferation and migration	[76]
miR-323-3p	Tumor suppressor	Down	Inhibits cell proliferation and EMT via modulation of SMAD2 and SMAD4 expression	[77]
miR-330-5p	Tumor suppressor	Down	Decreases cell proliferation and migration via inhibition of MUC1	[73]
miR-345	Tumor suppressor	Down	Proapoptotic	[65]
miR-506	Tumor suppressor	Down	Proapoptotic	[66]
miR-615-5p	Tumor suppressor	Down	Inhibits cell proliferation, migration, and invasion	[64]
miR-3923	Tumor suppressor	Down	Inhibits cell proliferation and viability via modulation of KRAS expression	[78]
miR-23a	Oncogenic	Up	Inhibits apoptosis and promotes proliferation and migration via inhibition of APAF1	[79]
miR-106a	Oncogenic	Up	Promotes proliferation, EMT, and invasion via targeting TIMPT-2	[80]
miR-181c	Oncogenic	Up	Promotes proliferation and cell survival via inactivation of HIPPO pathway	[81]
miR-191	Oncogenic	Up	Promotes cell proliferation via inhibition of USP10	[82]
miR-203	Oncogenic	Up	Promotes proliferation and migration via targeting of SIK1	[83]
miR-206	Oncogenic	Up	Promotes cell proliferation and invasion via induction of ANAXA2 and KRAS	[84]
miR-212	Oncogenic	Up	Cell proliferation and invasion through targeting of PTCH1	[85]
miR-221/222	Oncogenic	Up	Induces cell invasion via MMP-2 and MMP-9	[86]
miR-301a-3p	Oncogenic	Up	Invasion and migration via inhibition of SMAD4	[87]
miR-371-5p	Oncogenic	Up	Promotes cell proliferation	[88]

PDAC compared with chronic pancreatitis and healthy controls [90]. A training set selected 12 candidates (miR-16, miR-18a, miR-20a, miR-24, miR-25, miR-27a, miR-29c, miR-30a.5p, miR-19, miR-323.3p, miR-345, and miR-483.5p) for validation in four diagnostic panels in 137 subjects. In combination with CA19-9, one panel discriminated stage I and II PDAC from healthy controls (AUC 0.93, sensitivity 77%, specificity 94%). Further validation of this panel in combination with CA19-9 could lead to a clinically useful marker able to distinguish pancreatic cancer from chronic pancreatitis and healthy controls.

The exploitation of miRNA to detect early pancreatic neoplasia may offer the greatest potential to reduce morbidity and mortality. Current imaging features and tissue biomarkers obtained from invasive investigatory procedures are not sensitive enough to asses for malignancy of precursor lesions or to detect pancreatic cancer at an early stage of dysplasia. Recently next-generation sequencing in surgical tissue samples and endoscopic ultrasound-guided fine-needle aspirations (FNA) resulted in the identification of 40 miRNAs capable of discriminating premalignant intraductal papillary mucinous neoplasm (IPMN) and PDAC tissue from normal pancreas [91]. Validation in surgical samples ($n = 52$) and FNA ($n = 95$) showed the capacity of miR-103a, miR-155, miR-181a, miR-181b, and miR-93 to discriminate IPMN from controls with AUCs ranging from 0.68 to 0.92. Genome-wide miRNA profiling has further been employed to evaluate the reliability of miRNA signatures to differentiate low-risk/benign IPMNs from high-risk/malignant IPMNs in plasma in newly diagnosed individuals [92]. Five miRNAs, miR-200a-3p, miR-1185-5p, miR-33a-5p, miR-574-3p, and miR-663b, showed potential to discriminate between malignant and benign IPMNs (AUC 0.73, sensitivity 80.9%, and specificity 52.5%). The relatively small sample size and lack of validation sets limit the conclusions of this study. The findings do, however, support the need for further development of blood-based miRNA assays for IPMN diagnosis and management.

Most studies on miRNAs in pancreatic cancer have thus far been carried out with small patient and control numbers. Validation of promising miRNA candidates in independent, large cohorts will be necessary before being considered for clinical use. However, if data are reproducible when validated in large cohorts with all necessary controls integrated, circulating miRNAs may be a valuable resource for diagnosing pancreatic cancer.

As more diagnostic miRNA panels emerge, it will become increasingly important to ensure that data is reliable and open for meaningful interpretation. This will be achieved through careful optimization and standardization of sampling techniques and analytical methodologies. A great advantage of using miRNA as diagnostic tools is their increased stability compared to protein and mRNA and the feasibility of quantifying very low amounts of material from highly degraded samples [93]. Of additional benefit is their abundance in a variety of biological fluids and hence the potential for noninvasive testing. Measurement of miRNA has been achieved in most biological fluids including blood, urine, saliva, bile, and fine-needle aspirates [67, 91, 94]. To further advance the field, alternate sources of circulating miRNA should be investigated, such as extracellular vesicles and other tumor-derived carriers.

Extracellular Vesicle-Derived Markers

Extracellular vesicles (EVs) are a class of secreted membrane-derived vesicles which include exosomes, microvesicles, and apoptotic bodies. EV subpopulations are shed from numerous, if not all, cell types and have gained attention over the past decade for their role in both local and distant intercellular communication and disease pathogenesis. Exosomes are perhaps the most widely studied EV and will be the focus of this discussion, although it must be noted that other EV subsets, in particular microvesicles, are currently being investigated as sources of biomarkers of disease.

EVs are most often classified according to their size and mode of biogenesis. Exosomes are small EVs ranging in size from ~40 to 150 nm in diameter and originate from the inward budding of endosomal multivesicular bodies (MVB) within cells prior to their secretion. Structurally they are composed of a lipid bilayer which surrounds a cytosol devoid of normal cellular organelles. Exosomes, as with other subsets of EVs, are highly heterogeneous vesicles and can contain all known molecular constituents of a cell, including proteins, lipids, and nucleic acids [95, 96]. The contents reflect their cellular origin and are influenced by the physiological conditions in which they are generated and released. Once released from the surface of the originating cell, exosomal contents can be transferred to a recipient cell via fusion, where they are capable of mediating a phenotypic alteration in the cells which take them up [95]. It is this capacity to modulate the phenotype of recipient cells which makes exosomes key players in disease pathogenesis and which has led to a recent surge in interest for their characterization in a variety of disease states including cancer. Indeed, exosomes have been shown to hold the potential of carrying large arrays of oncogenic material from malignant to nonmalignant cells [97, 98], and studies have highlighted the roles of exosomal proteins and miRNAs in pancreatic cancer tumorigenesis, invasion, metastasis, and recurrence [99, 100].

Due to the close reflection of their cellular origin, exosomes released from cancer cells are a potential source of markers for the detection of cancer. The diagnostic value of exosomes is further evidenced by the variety of noninvasive sources for their collection, including blood, urine, saliva, and bile [101, 102], and the inherent protection they offer their cargo. Indeed, proteins, DNA, and miRNA have all been shown to be stable and abundant in exosomes [103, 104], with exosome-associated miRNA significantly more stable compared to free miRNA due in part to protection from RNase degradation [105]. Significantly higher exosome concentrations have been reported in the systemic circulation of patients with cancer versus controls [106, 107], and markers associated with cancer exosomes may therefore be enriched when harvested from heterogeneous populations of exosomes in biological fluids. The ExoCarta database (http://www.exocarta.org) holds an ever-increasing catalogue of proteins, lipids, RNA, and miRNA that have been identified in EVs from different sources.

Several studies have focused on developing exosome-associated biomarkers for pancreatic cancer detection. Recently, the heparin sulfate proteoglycan, glypican-1 (GPC-1), was reported as a highly specific exosome-associated biomarker for early detection of PDAC [107]. GPC-1 was measured in serum exosomes collected from

patients with PDAC (n = 190), benign pancreatic diseases (BPD) (n = 26), and intraductal papillary mucinous neoplasms (IPMN) (n = 5) and healthy volunteers (n = 100). The levels of GPC-1-positive (GPC-1^+) exosomes were found to be significantly higher in all 190 PDAC and 5 IPMN cases compared to BPD and healthy controls. Furthermore, GPC-1^+ exosomes revealed a sensitivity and specificity of 100% in distinguishing PDAC from healthy controls. These observations were consistent in a smaller validation study. Despite initial enthusiasm in GPC-1 as a breakthrough biomarker for early PDAC, it has also been shown to be associated with breast and colorectal cancer [107, 108], putting into question the test specificity. The diagnostic utility of GPC-1 was further questioned in a study by Lai et al. [109], where liquid chromatography-tandem mass spectrometry was employed to quantify GPC-1 levels in plasma exosomes. In a small cohort of PDAC, CP, and healthy control samples, no significant differences in GPC-1 levels were observed between the three groups. With further validation, GPC-1 may still hold value in the isolation of cancer-specific exosomes; however, current problems with the availability of specific GPC-1 antibodies will, at present, limit the utility of GPC-1.

Plasma levels of adrenomedullin (AM) have previously been reported in PDAC patients with diabetes compared to PDAC patients without diabetes and non-cancer subjects with diabetes [31]. Aggarwal et al. also established that pancreatic cancer (PCC)-derived AM inhibits insulin secretion by β-cells and AM was presented as a potential maker for type 3c (PDAC-associated) diabetes [31]. An independent study later demonstrated the release of exosomes by PCCs and their subsequent internalization by β-cells [110]. Western blot analysis confirmed the presence of AM in cancer-associated exosomes isolated from PCCs, and PCC-derived exosomes were further shown to inhibit insulin secretion in human islets, an effect abrogated by AM receptor blockade. These studies are important as they provide the first demonstration of a potential exosome-associated protein biomarker along with an associated function. Further validation will be required to establish whether exosome-associated AM provides an enriched source of AM for detection of type 3c diabetes among high-risk individuals.

As described above, in addition to exosomal protein biomarkers, exosome-associated miRNA may serve as diagnostic tools. The exosome provides a stable environment for miRNA and is a significantly enriched source compared to free-circulating miRNA [105, 109]. As such, it may be particularly advantageous when using miRNA for diagnostic purposes, to focus studies on the identification of cancer-associated miRNA located within exosomes. The miRNA content of human exosomes isolated from pancreatic cancer patients and control individuals has been studied by several groups. Que. et al. [111] found levels of miRNA-17-5p (miR-17-5p) and miR-21 to be heightened in serum exosomes from pancreatic cancer patients (n = 22) compared to healthy controls (n = 8), with good sensitivity and specificity for the diagnosis of PDAC (AUC 0.887, 95% CI: 0.796 to 0.978 and 0.897, 95% CI: 0.803 to 0.991, respectively). miR-21 was also shown to be expressed at higher levels in serum-derived exosomes from individuals with pancreatic cancer compared to those with chronic pancreatitis. Using a novel localized surface plasmon resonance (LSPR)-based sensor for specific and targeted miRNA

detection, Joshi et al. demonstrated the quantitative measurement (limit of detection ~10^{-9} M) of miR-10b in human plasma exosomes, pancreatic cancer cell lines, media, and human plasma [112]. Using their highly sensitive and specific sensing technique, the level of miR-10b was shown to be significantly elevated in plasma-derived exosomes from pancreatic cancer patients (n = 3) compared to chronic pancreatitis (n = 3) and normal controls (n = 3) (four- to tenfold increase and 50- to 60-fold increase, respectively). While this is a relatively small study in terms of sample size, Joshi and colleagues highlight the potential use of LSPR-based sensors, and other on-chip devices, in the label-free quantitative measurement of defined miRNA signatures within exosomes.

More recently, an enrichment of several previously reported cancer-associated miRNA was demonstrated within exosomes compared to whole plasma [109]. Using RT-qPCR, exosomal miR-10b, miR-21, miR-30c, miR-181a, and miR-let7a were shown to readily differentiate pre-resection pancreatic cancer samples (n = 29) from chronic pancreatitis and healthy control samples (n = 11 and 6, respectively). Interestingly, post-resection levels of miR-10b, miR-21, miR-30c, and miR-let7a showed a return to normal values, with a partial decrease in miR-181a observed. ROC analysis revealed that this group of five miRNA had 100% sensitivity and specificity in distinguishing pancreatic cancer from healthy controls.

The diagnostic complementarity of exosomal proteins and miRNA has been investigated in pancreatic cancer [113]. This research generated a pancreatic cancer-initiating cell (PaCIC)-specific marker panel selected via the analysis of exosomes isolated from PaClC culture supernatants compared with serum-derived exosomes from healthy controls. Candidate miRNA were selected via microarray analysis of exosomes isolated from PaCIC culture supernatant and serum-derived exosomes from pancreatic cancer patients and healthy controls. ROC analysis of both panels independently and in combination revealed their diagnostic complementarity. In a validation set comprising patients with pancreatic cancer, chronic pancreatitis, benign pancreatic tumors, non-pancreatic cancer malignancies, and healthy controls (n = 140 total), the combination of CD44v6, CD104, Tspan8, and EpCAM with miR-1246, miR-3976, miR-4306, and miR-4644 in serum-derived exosomes showed excellent sensitivity (100%, 95% CI: 0.95 to 1) with a specificity of 80% (95% CI: 0.67 to 0.90) for PDAC versus all other control groups. Excluding other malignancies, specificity reached 93% (95% CI: 0.81 to 0.99). Interestingly, miR-1246 and miR-4644 have also been shown to discriminate PDAC from healthy controls in salivary exosomes (AUC 0.83) [114], opening up the potential future validation of miRNA, either alone or in combination with protein markers, in a variety of biological fluids.

The noninvasive methods of sample collection, sample stability, and diverse cargo which reflects their cellular origin make exosomes, and EVs in general, an attractive source of biomarkers. However, to date, a highly specific pancreatic cancer diagnostic marker has not been fully validated in EVs. It is expected that the number of studies identifying EV-associated biomarkers will continue to rise. As such it is important to address the current shortfalls in EV analysis which will limit the meaningful interpretation of validation studies. Any EV study must address the

heterogeneity of the EV population analyzed and must demonstrate the association of the function or feature attributed to the EV of interest by specific co-isolation. Where other vesicles have the same functions, the whole component of EVs must be analyzed, not just a specific subset (e.g., exosomal) fraction. Where a biomarker is associated with a specific EV subset, strict standards must be applied to correctly evidence the isolation methodology (e.g., via surface markers or size distribution).

The present lack of standardized methods for the isolation and characterization of EVs significantly hinders their potential as routine clinical markers. However, in response to the rapid increase in interest of EV, there are an increasing number of commercial kits for isolation and purifications, and a number of analytical tools, such as lab on a chip, are being investigated [115, 116]. Coupled with the efforts of EV working groups to standardize workflows and the distribution of guidelines such as the minimal information for studies on EVs (MISEV) [117], it is likely that EV-associated markers will in the future become clinically viable diagnostic tools.

Imaging

Multiple detector computed tomography (MDCT) has proven to be the optimal modality for staging of pancreatic masses, with magnetic resonance imaging (MRI) and endoscopic ultrasound (EUS) complementing it. MDCT has high sensitivity and specificity to predict resectability of pancreatic tumors and detects focal metastasis. MRI has superior sensitivity to identify small lesions, characterize parenchyma, and delineate the relationship of lesions to the ductal system. Certainly when monitoring of cystic lesions is required, MRI is preferred due to lack of repeated exposure to radiation. EUS is being increasingly used as it can detect small focal lesions and allows sampling for cyto-molecular analysis [118]. Fludeoxyglucose F-18 positron emission tomography (FDGPET) detects the increased metabolic activity of tumor cells and can identify small metastasis and differentiate cancer from benign cystic lesions [119]. It can identify sub-centimeter lymph node metastases, but reports suggest its sensitivity is affected when lymph nodes are in close proximity to the pancreas, and a high false-positive rate in hyperglycemic patients must be addressed [120]. Nevertheless, it is used to identify occult metastasis and also assess response of tumors to adjuvant therapies.

Novel technologies are being utilized to identify early pancreatic cancer and predict malignant transformation of IPMN. Early recognition of malignant lesions is thought to increase the chances of curative resection and hence improve overall survival. Magnetic resonance imaging (MRI), magnetic resonance cholangiopancreatography (MRCP), and EUS can quantify IPMN lesions, but they are not able to identify PanINs nor can they grade IPMNs. Dual-energy computed tomography (CT) has been shown to have increased sensitivity in detecting lesions of less than 2 cm and also iso-attenuating tumors, which make up some 10% of pancreatic tumors. Hybrid PET/MRI is being trialed for staging response to adjuvant/neo-adjuvant therapy. Fluorothymidine positron emission tomography (18F–FLT PET) is a new molecular imaging modality targeting the proliferative activity of tumor

cells and was shown to have increased specificity compared to other imaging modalities [121]. EUS is being increasingly utilized to assess pancreatic parenchyma and cystic lesions and allows relatively easy access to the collection of tissue biopsies or cystic fluid for analysis of protein markers and genomic studies. The rapid advancement of imaging techniques has led to improvements in earlier diagnosis of pancreatic cancer. With new technologies currently in development, there is great potential for diagnosis of high-risk pancreatic lesions and PDAC at earlier stages, leading to improved patient outcome.

Emerging Technologies

Metabolomics

Metabolomics is the most recently established "omics" strategy employed in systems biology. It describes the study of metabolites in biological systems and most often involves the quantitative determination of low molecular weight metabolite concentrations, both at a system-wide and at a cellular level. The human metabolome is the ultimate product of a process originating with the genome. Compared with other omics strategies, such as genomics and proteomics, metabolomics offers the greatest potential to observe the phenotype of the system. The metabolome is extremely responsive to varying physiological conditions and, as such, can provide a snapshot of the biological state at specific time points. Research has shown that metabolic reprogramming is one of the hallmarks of cancerous cells and many of the genes and proteins found to play important roles in cancer are known to be involved in metabolic processes. Metabolic profiling holds great potential as a powerful tool for the discovery and development of clinically viable biomarkers.

Metabolomics strategies chiefly employ either targeted (hypothesis-driven) or untargeted (hypothesis-generating) approaches. Targeted strategies involve the assessment of a defined number of metabolites, or a specific metabolic pathway, and benefit from maximum analytical sensitivity and specificity. Untargeted strategies aim to capture all metabolic pathways and the maximum number of metabolites under a given set of experimental conditions. This untargeted workflow comes at the cost of lower analytical sensitivity and specificity. A variety of analytical techniques are utilized to facilitate these approaches, including proton nuclear magnetic resonance (^{1}H–NMR) spectroscopy, gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and Fourier transform-ion cyclotron resonance-mass spectrometry (FT-ICR-MS). Increased capabilities of state-of-the-art techniques offer the benefit of both targeted and untargeted analyses in the same analytical run. As with other omics workflows, sophisticated bioinformatics tools are required to process the vast data sets generated and to explore biologically significant findings. Multivariate analyses, such as principal component analysis, partial least squares analysis, cluster analysis, and random forest, are most commonly employed for visualization and interpretation of global data sets, essential in biomarker discovery workflows. By contrast, univariate analysis has utility in the

discrimination of individual metabolites, which is particularly useful in secondary biomarker analyses.

Despite the firm standardization now routinely implemented in other omics fields, such as proteomics and genomics, metabolomics studies often fail to reach necessary analytical standards. Importantly, metabolomics studies frequently fail to include relevant control groups and the validation of findings in at least one independent cohort of samples. The use of matched pre- and post-diagnostic samples is also critical if biomarkers capable of distinguishing pancreatic cancer at an early stage are to be discovered. Finally, appropriate sample collection and storage are critical for the meaningful interpretation of metabolomics data. The influence of circadian rhythm, diet, xenobiotic exposure, and underlying physiological conditions must be considered along with efforts to quench ongoing metabolism post-collection.

An overview of findings from recent comprehensive metabolomics-based biomarker research in pancreatic cancer is presented below.

A targeted UHPLC-MS/MS method employing a commercial 206 metabolite data set utilized sparse partial least squares discriminant analysis and greedy stepwise and GeneticSearch algorithm to select four metabolites with high discriminating potential [122]. Of these, palmitic acid and oleanolic acid exhibited excellent diagnostic accuracy (AUC 1.0) when subjected to ROC analysis in the cohort of PDAC (n = 40) and HC (n = 40) serum samples. At a cutoff value of 134.3 μM, palmitic acid outperformed CA19-9, achieving 100% sensitivity and specificity. Future validation in large cohorts with relevant controls would be required to further investigate the clinical utility of palmitic acid in pancreatic cancer. However, this discovery study clearly demonstrates the potential of multivariate methods and classification trees in the interrogation of MS-based metabolomics data.

Richie et al. [123] generated comprehensive metabolic profiles of sera from pancreatic cancer patients and healthy controls using a nontargeted approach on a FT-ICR-MS platform. A significant alteration in the metabolome of pancreatic cancer patients was shown with alterations in a number of metabolites including long-chain fatty acids, cholines, and sphingomyelins. The ultra-long-chain fatty acid PC-594 was subjected to further validation, along with CA19-9, in an independent cohort (n = 188) [124]. ROC analysis revealed the superior performance of P-592 compared to CA19-9 in distinguishing pancreatic cancer from normal controls (AUC of 0.93, 95% CI: 0.91 to 0.95 and 0.85, 95% CI: 0.82 to 0.88, respectively). A PC-594 threshold of 1.25 μmol/L produced a relative risk (RR) of 9.4 ($P < 0.0001$, 95% CI: 5.0 to 17.7), sensitivity was 90%, and specificity was 87%.

Accurately distinguishing pancreatic cancer from chronic pancreatitis remains a significant challenge in terms of correctly diagnosing pancreatic cancer. Recently, a metabolite-based biomarker signature was identified that can discriminate pancreatic cancer from chronic pancreatitis with much greater accuracy than is currently observed with CA19-9 alone [125]. In a study involving more than 900 subjects, 477 blood-based metabolites were identified with 29 of those significantly altered in level between pancreatic cancer and chronic pancreatitis patients. A nine metabolite signature was derived and when used with CA19-9 showed very high accuracy for the discriminating pancreatic cancer and chronic pancreatitis [125].

A large proportion of metabolomics studies in pancreatic cancer have focused on glucose and glutamine metabolic pathways; however, there is increasing interest in the diagnostic potential of altered amino acid metabolism. A large study conducted in Japan [126] employed targeted LC-MS/MS to the analysis of 19 plasma-free amino acids (PFAA) in 360 pancreatic cancer patients, 28 chronic pancreatitis patients, and 8372 healthy controls. In fasting plasma samples, 14 PFAA were shown to be differentially expressed in pancreatic cancer ($p < 0.05$). A multivariate model using six specific PFAA (serine, asparagine, isoleucine, alanine, histidine, and tryptophan) was developed and applied to training (PDAC n = 120, HC n = 600) and validation (PDAC n = 240, CP n = 28, HC n = 7772) sets. ROC analysis of the PFAA index showed good sensitivity and specificity for the diagnosis of pancreatic cancer in the training set (AUC 0.89, 95% CI: 0.86 to 0.93). Validation of the PFAA index continued to demonstrate good sensitivity and specificity in the distinction of pancreatic cancer patients from healthy controls and patients with chronic pancreatitis (AUC 0.86, 95% CI: 0.84 to 0.89 and 0.87, 95% CI: 0.80 to 0.93).

Most biomarker studies employ a cross-sectional design, comparing samples collected at a single time point after diagnosis. This approach limits the observation of molecular changes that occur early in disease progression and hence the identification of markers for early diagnosis. To investigate whether global metabolic changes could be detected in circulating metabolite levels in the years preceding pancreatic cancer diagnosis, Meyers et al. [127] profiled metabolites in pre-diagnostic plasma from individuals with pancreatic cancer and matched controls. Levels of three branched-chain amino acids (BCAAs), leucine, isoleucine, and valine, were strongly associated with future PDAC development ($p \leq 0.0006$). Individuals with the highest BCAA levels had at least a twofold increased risk of developing pancreatic cancer, with the risk greatest 2–5 years prior to diagnosis. Interestingly, while elevated BCAA levels are also associated with diabetes [128], a risk factor for PDAC, the correlation between BCAA levels and PDAC risk was found to be independent of diabetes.

A number of metabolomics-based biomarker signatures have been proposed for the diagnosis of pancreatic cancer; however, none have moved beyond the discovery phase. With the increasing application of high-resolution MS-based methodologies, there is the promise of more comprehensive coverage of the metabolome and ultimately the creation of robust biomarker panels with clinically viable sensitivity and specificity.

Conclusion

While a number of biomarkers for PDAC have now been reported in the literature, significant efforts are still required to fully validate their clinical utility in the early detection of PDAC. Both biomarker discovery and validation programs require large numbers of samples. Increasingly, it is recognized that collaboration between specialist centers is required to achieve the samples necessary for robust studies. Sampling from different centers also rules out local bias in collection method.

In addition, longitudinal patient sampling is not frequently undertaken. Thus, although samples are taken at the time of diagnosis, enormous value could be derived if biomarker levels were measured again following surgery and chemotherapy. The use of high-risk registries will provide both the samples required for discovering biomarkers and the individuals in which to test good candidate markers. Understanding the relationship between type 3c diabetes and PDAC may contribute to the early detection of PDAC. However, this will require the collection of samples from individuals newly diagnosed with diabetes and the recognition of the importance of this high-risk group in research groups in Europe as it is currently in the United States.

Good practice, such as the use of training and test sets that are independent; the careful choice of samples, with variables such as age and gender matched across comparator groups; and the avoidance of known confounding factors and of overfitting of data [129] should all contribute to higher-quality studies. Careful review of manuscripts and judicious editorial decisions should prevent biomarkers that have already been discounted from being published yet again as promising candidates.

Finally, significant improvement in the survival of pancreatic cancer patients will only come about if progress in early detection is concurrent with advances in treatments.

Cross-References

- ▶ Approaching Pancreatic Cancer Phenotypes via Metabolomics
- ▶ Cancer Exosomes for Early Pancreatic Cancer Diagnosis and Role in Metastasis
- ▶ Circulating Tumor Cells
- ▶ Diagnostic Biomarkers
- ▶ Familial Pancreatic Cancer
- ▶ MRI and MRCP for Diagnosis and Staging of Pancreatic Cancer
- ▶ Pancreatic Adenocarcinoma: CT and PET/CT
- ▶ Secondary Screening for Inherited Pancreatic Ductal Adenocarcinoma

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Development of Novel Therapeutic Response Biomarkers

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Contents

Abstract

Biomarkers that can indicate the best treatment option for each patient could greatly improve pancreatic cancer survival. Markers need to be practical to use in a timely fashion in order to change the choice of therapy. In vitro or ex vivo studies are useful in identifying potential markers, but these may not have relevance to marker profiles of in situ tumors, and adequate quality of tumor tissue may not be routinely available in patients with advanced disease, and so

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blood-based markers of systemic determinants of response may be more attractive. Any marker, tissue- or blood-borne, needs to be tested in clinical studies involving multiple populations before entering routine use. These studies cannot rely just on prognosis as one individual's survival may be improved by therapy but still be significantly shorter than another whose survival was independent of therapy. Ideally an objective measure of response that links to survival benefit should be used to evaluate a biomarker. However, this may not be possible for adjuvant therapy where the tumor is removed before treatment begins and the link between survival and response in an advanced setting is not always reliable. Survival on its own is a poor surrogate for response, and its use may lead to confusion of prognostic and response markers unless used within large clinical trials. Adverse responses to treatment such as rash linked to survival may be an alternative measure. Difficulties in defining the level of beneficial response make empirical identification of response biomarkers difficult. Theory-based studies have more power to identify and validate markers, but the determinants of drug response are complex, and popular (but potentially misguided) beliefs about specific proteins may lead to multiple testing and hence type 1 errors. Grouping biomolecules (proteins, RNA, metabolites, or DNA sequences) into marker panels linked to function, for example, grouping proteins that determine mesenchymal transition of cancer cells or which define the nature of stroma, may offer a way forward. Alternatively, functional analysis alone, including level of immune response, may allow the most beneficial therapy to be directed to each patient.

Keywords
Gemcitabine · 5-FU · Capecitabine · Tegafur · DPD · Thymidylate synthase · Ribonucleotide reductase · hENT1 · CDA · Response · Survival

The Promise of Personalized Medicine

It is not as easy to be defeatist when discussing pancreatic ductal adenocarcinoma (PDAC) as it once was. Clinical trial after clinical trial has shown that long-term survivors do exist [1, 2] and that the proportion of these survivors will depend on the specific treatment regimen chosen. Most patients will not respond or will only have a transient benefit, but in the last few years, it has become apparent that populations can be identified who will benefit from one treatment but not another [3]. It may even be true that one individual's cure may be another's poison, making their individual prognosis worse than it would have been with no treatment at all. New treatment modalities are being developed, in particular immunotherapy and targeted therapy [4], and although the benefits have yet to live up to the initial expectations for these agents, they join a well-established range of chemotherapeutics with ever-improving regimens that are achieving iterative (albeit small) improvements in survival. What we lack are the tools to identify which therapy to give to which patient and when to change the therapy as the cancer evolves.

Requirements of a Therapeutic Biomarker

Diagnostic biomarkers help clinicians to diagnose the presence and origin of the disease or the relapse of a previously treated disease that was in remission. Prognostic biomarkers discriminate between patients with the same cancer diagnosis who will do better or worse. Whereas therapeutic biomarkers indicate the benefit of a certain treatment, this can be predictive of the cancer's response or the patient's tolerance to a certain therapy.

In some cases, a biomarker can be classified in more than one of these categories, e.g., prostate-specific antigen (PSA) may be used in diagnosis, in early detection of relapsing disease, and as a prognostic marker in assessment/follow-up of given therapies [5]. Another example is the amplification status of HER2 in breast cancer; this is of general prognostic value (HER2-positive patients have worse prognosis than HER2-negative ones) [6], but beyond this, it is also predictive of treatment response (i.e., HER2-targeting treatments will only benefit HER2-positive patients) [7]. In some cases, the relationship between a biomarker and prognosis can be the inverse of its relationship to response, for example, high levels of the protein HuR (which will be discussed later in this review) are in general associated with poor survival but also good response to the drug gemcitabine [8].

Therapeutic response can be measured in a number of ways. For neoadjuvant therapy and in advanced cancer, it can be measured by reduction of tumor volume assessed by imaging techniques, reduction in tumor biomarkers, or increased time to progression. With adjuvant therapy, the target is micrometastatic disease which is not measurable by any standard means, so it is not possible to measure any reduction in tumor volume, and (to add a further complication) the tumor, which is the most likely source of therapeutic biomarkers, is removed before chemotherapy begins. For all therapy, overall survival is the most important measure, although it is difficult to separate prognosis and response on this basis.

In order to enter clinical practice, a therapeutic biomarker has to pass a series of hurdles as illustrated in Fig. 1. A marker must be selected where a biomarker-positive patient gains more benefit from a given treatment than patients who are negative for that marker. This is not the same as marker-positive patients having a survival advantage over marker-negative patients; in fact marker-negative patients could have better overall survival than patients who are marker positive but still be getting less benefit from the therapeutic: it is necessary to show that the survival benefit is treatment specific. Even if a biomarker indicates greatest benefit from one drug, it will not necessarily be of any practical use, knowing that the benefit patient A gets from, for example, gemcitabine is less than the benefit patient B will receive is of little comfort to patient A if gemcitabine is the only option for treatment. It is self-evident that to be of greatest use, a therapeutic marker should allow a choice of therapeutic, so a negative result for one therapy should indicate that a patient would get more benefit from an alternative therapy. In the absence of an alternative treatment, the only utility the biomarker can have is to indicate the absence of any benefit, thereby allowing a toxic drug to be avoided. This is a very real advantage, but depressingly in the case of pancreatic cancer, this advantage may be associated with the loss of the last hope a patient has.

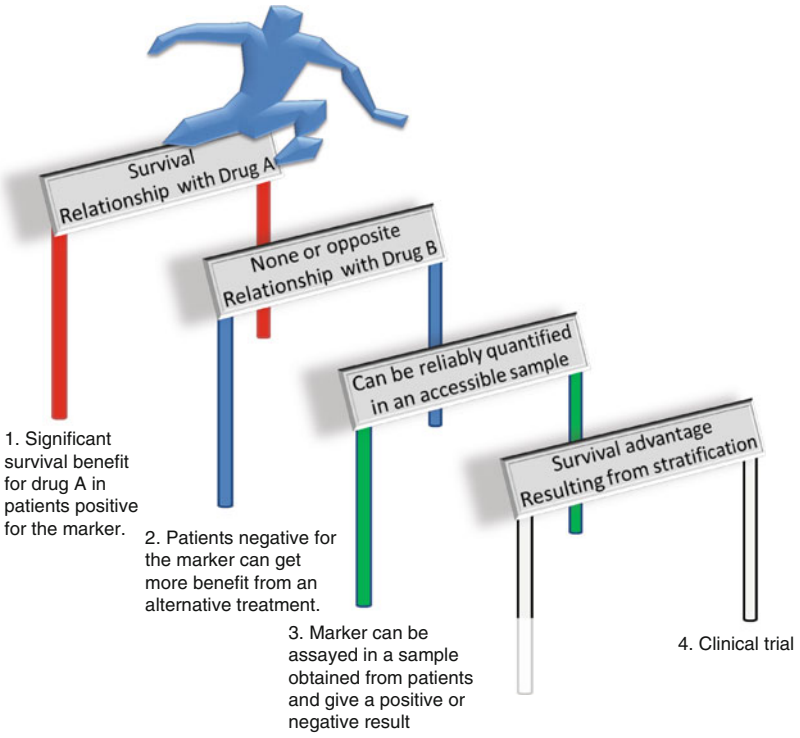

Fig. 1 Hurdles that must be overcome to get a therapeutic biomarker to the clinic. In order for a therapeutic marker to be useful, it must not only indicate individuals who benefit from a therapy but also indicate that they would benefit less from an alternative. It must also be practical to adequately quantify the marker. Only then is it suitable for clinical trial

In order to reach the clinic, the biomarker should be validated in a clinical trial, but before this can happen, it must be possible to apply the biomarker in a clinical setting. There is surprisingly little guidance on what is required in identifying and utilizing a biomarker for therapeutic response. The problems associated with testing a biomarker in a clinical trial are somewhat different from testing a drug. A fundamental difference to pharmacokinetic analysis is that the test substance is indigenous. The Crystal City VI meeting in 2015 addressed this issue [9], concluding, among other things, that spiking as a method to measure linearity of measurements and limits of quantification is generally unsafe. To overcome this, clinical samples with high levels of biomarkers should be diluted in an appropriate matrix (e.g., serum or an artificial equivalent). Analysis of parallelism (measured concentration change related to theoretical concentration change) can be used to define adequacy of an artificial matrix, and if an artificial matrix can be validated, it could potentially be used to assess a measure of assay accuracy; otherwise, thresholds for biomarkers will have to be admitted to be pragmatically defined.

Even when a marker can be validated in a research project, it may be impractical in the clinic due to availability of adequate clinical samples. Germline DNA is

unlikely to be a problem as blood, saliva, or buccal swabs can be obtained from any patient, and thanks to the polymerase chain reaction, tiny amounts are adequate for even the most complex analysis (even whole-genome sequencing). However, germline differences are very unlikely to explain the majority of variation in drug response given the far greater genetic variation in different tumors compared to different germlines. Identifying tumor-specific differences in blood samples is very attractive, but distinguishing differences due to the cancer from background variation may be a challenge and require relatively large volumes of blood that may be difficult to obtain from very sick patients, for example, obtaining sufficient numbers of circulating tumor cells for intracellular protein or RNA analysis may be feasible in some but not all patients. Even for patients undergoing curative intent surgery, heterogeneity of the sample may be a significant practical problem, convenient approaches such as the use of tissue microarrays may work where it is possible to reconstruct a 3D model of the original tumor, allowing the relevant cancer or stromal regions to be identified, and this may be possible within a research study, but in routine care, the clinician may have to rely on data from one or two cores per patient. For patients presenting with inoperable and/or metastatic disease, large volumes of tumor tissue are rarely available. In the best case, representative biopsies can be taken, but in current practice, fine-needle aspirates are more common (in many cases, diagnosis relies exclusively on radiology and clinical signs), so a biomarker validated in biopsy samples may not be applicable without a very dramatic change in clinical practice.

Identification of Biomarkers

There are a variety of ways by which candidate biomarkers are identified, but in essence, these can be divided into two classes: empirical and theoretical. The theoretical approach has the disadvantage of being much more sensitive to publication bias. Each analysis will be based on a restricted number of hypotheses (e.g., the hypothesis that marker A is associated with response); therefore, the statistical barrier to demonstrating an association will be relatively low (e.g., $P<0.05$). However, if a theoretical relevance is clear, then it is likely that many groups will look for an association, and failure to show an association will be difficult to publish, but if this is tested enough times, sooner or later significance will be suggested and will be relatively easy to publish. In this respect, empirical analysis is safer, but the barrier to establishing a relationship with response is obviously much greater.

Empirical Analysis

Intuitively the best way to identify a biomarker of response is simply to detect something that is present in those patients who respond to the therapy and is absent in those who do not (or vice versa). Of course, if enough potential markers are

examined, positive results will be found, but this problem can be overcome by rigorously testing independent populations after the initial discovery process: so all that is needed is multiple populations of responders and nonresponders. However, in the case of pancreatic cancer, identifying a responder is not as simple as it sounds. In an adjuvant setting, there should not be any visible tumor after resection so imaging cannot be used to measure response to a drug. Theoretically, biomarkers produced or induced by metastases could be used instead, but this depends on the marker being stably detectable after resection (if a marker such as CA19-9 declines to extinction after resection, regardless of treatment, its decline cannot be taken as an indication of response in a given individual). Survival is a poor surrogate as a long-term survivor might have done even better without the drug and poor survivors may contribute to an overall clinical benefit by surviving longer than they would have done otherwise.

Responders

In an advanced setting, there is a tumor mass to follow, but reduction in tumor mass does not necessarily equate to a survival benefit. Changes in volume of primary tumors may, for example, not reflect changes in more critical but difficult to measure metastases. Also clinically important responses such as necrosis or metabolic changes may be missed, while reduction in tumor volume due to reduced stroma may be difficult to distinguish from a genuine reduction in cancer cells within the primary. With immunotherapy in metastatic malignant melanoma and other solid cancer, the caveat of "pseudoprogression" has been highlighted [10]; this means that the tumors grow in volume on radiology scans and are mistakenly interpreted as progressive disease, whereas the true reason is not growth of tumor cells per se but recruitment of immune cells doing their job attacking the cancer. In a Japanese study using Response Evaluation Criteria in Solid Tumors (RECIST), it has been shown that progressive disease does correlate with worse survival in advanced pancreatic cancer, but no significant survival difference was seen between stable disease and partial response using the same criteria in patients receiving chemotherapy [11]. This seems inconsistent with data from other solid tumors where an almost linear correlation exists between reduction in tumor size following chemotherapy and survival [12]. Whether this indicates a difference in tumor biology or study design is difficult to know.

Conceptually, a reduction in CA19-9 would seem an attractive measure of response (assuming this is a specific measure of metastatic and primary cancer burden). However, a drop in serum CA19-9 levels in patients with advanced pancreatic cancer after treatment has been shown to be a poor indicator of prognosis following chemotherapy [13].

Neoadjuvant therapy offers a much clearer association between objective measures of response and survival, with the added advantage that such patients provide tissue samples that can be used to identify and test potential biomarkers. Measures such as tumor regression grade (TRG) [14] and reduction in CA19-9 levels [15] indicate better survival. To an extent this association with survival is implicit, as neoadjuvant therapy has as yet been confined to patients who are borderline

resectable, so measures of response define the patients who will go on to receive surgery and who, for that reason, will be likely to survive better.

Ex Vivo Analysis

As an alternative to survival as a measure of response, tumor cells can be removed from a patient and treated with therapeutics outside of the body. Clearly, this removes many potential factors that determine response in the actual patient: the immune system, tolerance of the patient for the agent, the tumor microenvironment, 3D interactions of tumor cells with stromal cells and with themselves, etc. To address some of these issues, patient-derived xenografts (PDX) [16] and organoids [17] have been employed to test response. Treating such models with drug and measuring growth inhibition and cell death could in theory be used in itself as a marker for response in the patient, unfortunately the time required to get a result means that this would be difficult to apply clinically. The approach is more easily applied in identifying potential biomarkers expressed in the isolated cancer cells.

Survivors

Many studies have identified prognostic markers that have then been associated with response to chemotherapy based on multivariable analysis. It can be questioned whether these are truly empirical studies (i.e., was the choice of variables to include influenced by theoretical considerations of drug action) but where the data was collected as part of normal clinical practice, this can at least be defined as semiempirical. Some studies have simply used the observation of a biomarker's association with improved overall or progression-free survival in a cohort of patients treated with chemotherapy as evidence that the biomarker relates to response. In this way, markers such as derived neutrophil-lymphocyte ratio have been claimed to be linked to the effectiveness of chemotherapy regimens including gemcitabine [18]; supporting studies will be described later that make the same conclusion based on very clear theoretical considerations.

Genome-wide association studies suggested that the single-nucleotide polymorphism (SNP) rs11644322 is associated with gemcitabine-specific outcome [19]. This SNP is within the gene for WWOX, which inhibits Wnt signaling upstream of β-Catenin. The A allele of rs11644322 binds SP family members more tightly than the more frequently occurring G allele (allele frequency approximately 75%) resulting in lower expression of WWOX, which has been associated with poorer survival in PDAC patients treated with gemcitabine [19]. Knockdown of WWOX gives greater gemcitabine sensitivity in lymphoblastoid and pancreatic cancer cell lines but did not alter sensitivity to 5-FU significantly. Lymphoblastoid cell lines with GG have lower gemcitabine EC50 levels than the AA and GA versions.

Low levels of the E3 ligase CBL relates to poor survival [20]. CBL also seems to relate to chemoresistance in cell lines, and it is proposed that it helps regulate ERB2 such that its impact on chemoresistance may be modified by the use of erlotinib [21].

High level of cancerous inhibitor of protein phosphatase 2A (CIP2A) has been related to poor survival (although no data was given on chemotherapy), and knockdown of CIP2A in cell lines was claimed to increase sensitivity to gemcitabine [22].

A biomarker that predicts survival benefit with a treatment yet indicates no survival benefit without the said treatment is indicative of a relationship with response. This can even indicate a subpopulation of patients who will benefit from a treatment that overall gives no benefit. For example, bevacizumab did not offer any survival advantage when used in combination with gemcitabine over gemcitabine alone, while abnormal pretreatment serum albumin levels were associated with poor survival in patients treated with bevacizumab but had no benefit in patients treated just with gemcitabine (the implication being that patients with normal b-albumin should receive bevacizumab) [23].

Omic Categorization of Tumors

Tumors can be categorized on the basis of their genome, transcriptome, or metabolome. Response measures can then be compared across the groups.

Whole-genome sequencing of 100 PDAC samples combined with analysis of copy number variation indicated four subtypes of cancer: stable, locally rearranged, scattered, and unstable [16]. Of the five patients who were either in the unstable group or who had otherwise been defined as "on-genotype" due to association with BRCA pathway mutations, four had at least partial response to platinum-based therapy, compared with none of three in the "off-genotype" group. This was supported by two of three "on-genotype" PDXs responding to cisplatin, compared with none of four "off-genotype" PDXs.

Collisson et al. used transcriptional profiling to divide PDAC tumors into three subtypes: classical, quasi-mesenchymal (QM), and exocrine-like [24]. Subtype-dependent in vitro responses to gemcitabine and erlotinib (an EGFR-targeting tyrosine kinase inhibitor) were revealed, with QM being more sensitive to gemcitabine and the classical subtype being more sensitive to erlotinib. It remains to be proven whether the multigene profile is predictive in patients.

To date there has been limited progress in categorizing PDAC based on different metabolomes, but it has been possible to identify a metabolic profile that distinguishes PDAC from pancreatic parenchyma, and within this profile, high cancer ethanolamine was associated with worse survival [25]. In liposarcomas, metabolic profiling of cell lines derived from PDXs distinguished cell lines that responded to gemcitabine from those that did not. The basis of this metabolic difference appears to be high expression of deoxycytidine kinase which increased nucleoside uptake by cells in culture. This could be measured in vivo using positron emission tomography with 1-(2′-deoxy-2′-[18F]fluoroarabinofuranosyl) cytosine (FAC).

Adverse Response to Treatment

An adverse response may limit effectiveness of a given agent; the side effects themselves may be a cause for discontinuation of treatment or dose reductions to the point where any potential benefit is lost. On the other hand, an "adverse response" can be evidence of activity and so could be linked to a survival benefit. For example, some chemotherapeutic and immunotherapy agents cause a rash as an adverse event [26, 27]. In some cases, it has been observed that patients with

a rash have better survival than patients without: this was shown in response to combined gemcitabine or capecitabine with erlotinib [28], and it was also shown with a combination of cetuximab, gemcitabine, and oxaliplatin (followed by chemoradiation with cetuximab) [29]. On the other hand, no relationship was seen between rash and survival with a combination treatment of erlotinib and capecitabine in gemcitabine refractory patients [30].

Rash as a marker of response has a significant disadvantage in that it can only be assessed after the drug has been administered. Furthermore, it takes time to develop, and so a potential window for using an alternative therapy could be lost. There have been attempts to link the development of rash to genetic factors and other biomarkers. Overexpression of EGFR was found not to be linked to rash [31]. On the other hand, adverse events (including rash) associated with gemcitabine have been suggested to be linked to a deleterious cytosine deaminase polymorphism [32], and adverse events associated with capecitabine (including one case of rash) have been reported to be more prevalent in patients with variants in the thymidylate synthase gene enhancer [33].

Resistant Cell Lines

The question of multiple drug resistance and specific drug resistance can also be addressed by generating resistant cell lines. Empirical comparison of expression, mutations, or epigenetic changes between resistant and nonresistant lines can then be addressed, the problem being that this cannot easily identify determinants of response that require interaction between cancer cells and stroma or which are systemic in nature.

A study of acquired gemcitabine resistance in ten derivatives of the cell line BxPC3 concluded that there was little cross resistance because only one cell line had acquired resistance to all the other agents they tested (5-FU, CDDP, CPT-11, and DTX). However, there was evidence of cross resistance to at least one agent in nine out of ten of the lines, so depending on the perspective of the reader, this could be viewed as either proof for or against acquired multidrug resistance. The authors explain gemcitabine resistance in at least four of the cell lines on elevated transcript levels of one of the components of the gemcitabine target ribonucleotide reductase, RRM1. This empirical discovery was perhaps strongly influenced by theory, but other genes involved in gemcitabine transport (human equilibrative nucleoside transporter 1, hENT1) and activation (deoxycytidine kinase, dCK) were excluded as their mRNA was not increased. This assumes transcript level equates to protein level, and the authors provide support for this in relation to RRM1 [34].

Cell lines have also been used in an attempt to identify intrinsic drug resistance, for example, Kim et al. applied elegant proteomic analysis of the cell lines BxPC3 and Panc1 on the basis that the latter cell line is more resistant to gemcitabine and therefore markers that distinguish the cell lines could be markers for resistance [35]. The approach used cannot truly be described as empirical as the differences highlighted were selected on the basis of a theoretical relationship with resistance. Specifically, upregulation of genes associated with epithelial mesenchymal transitions (EMT) was taken to relate to drug resistance because of previously described association between EMT and gemcitabine response [36].

Regulatory Factors as Response Markers

Before discussing methodologies based on known pathways of drug metabolism and action, it is worth considering studies that have focused on regulatory factors selected on observed measures of response rather than on the assumed target of regulation, the most obvious example being microRNA (miRNA) analysis. miRNA arrays or next-generation sequencing techniques have been used to identify individual species or panels of miRNA from resected tissue which correlate with prognosis; these have then been mapped back to the mRNA they regulate in order to propose mechanisms for survival advantage [20]. miRNA species can also be analyzed in a similar way from plasma or serum; comparison between profiles from cancer and noncancer patients can be used to indicate cancer-specific species [37]. On the basis of such profiles, associated with prognosis and/or tumorigenesis, specific miRNA species have been selected and examined for a role in treatment-specific response (using specific PCR or sequencing methods to quantify individual species). In particular, miR-21 has been proposed as a response marker on the basis of low expression being associated with benefit from adjuvant therapy (including gemcitabine) [38]. Association of other miRNAs with drug response has been heavily influenced by assumed mechanisms, for example, the assumed role of ribonucleotide reductase in determining gemcitabine resistance extends to an assumed role of miRNA 101-3p which has been shown to reduce the levels of RRM1 and to restore chemosensitivity to pancreatic cancer cell lines that have acquired chemoresistance due to RRM1 overexpression [39].

If analysis of miRNA is an attractive area for investigation of drug response due to their pleiotropic destabilization of RNA species, factors that stabilize mRNA must similarly be of interest. The protein HuR binds to the 3′ untranslated regions of specific mRNA in response to stress, stabilizing these transcripts. The protein has been shown to be upregulated in various forms of cancer [40], and cytoplasmic localization is associated with poor prognosis [41]. However, low (not high) expression of HuR was found to associate with poor survival in pancreatic cancer patients treated with gemcitabine [8]. HuR was shown to increase levels of dCK and increase sensitivity to gemcitabine in cell lines, which could explain why it increases the sensitivity to gemcitabine (increasing the level of the active metabolite trapped in cancer cells). It could also partly explain why cancer aggression increases in the absence of gemcitabine by potentially increasing the level of nucleotides available for rapidly dividing cells [8]. However, in the RTOG trial, 9704 dCK levels were found to be associated with good survival in patients treated with 5-FU, and, although cytoplasmic HuR did correlate with dCK, no survival advantage was seen with HuR itself [42]. The authors of this study explained the lack of association between survival and HuR on radiation interfering with HuR's regulatory effects [42].This confusing story is perhaps instructive and reflects the difficulty of assessing the impact of proteins which have multiple effects and are affected by multiple factors. Chemotherapeutics including gemcitabine, but also including mitomycin C, oxaliplatin, cisplatin, carboplatin, and a PARP inhibitor, cause HuR to migrate from the nucleus to the cytoplasm, and so measurement of HuR posttreatment will give a very different impression than pretreatment measurement [43]. It is also perhaps a little too easy to

choose the HuR effect that best fits with our assumptions, dCK is regulated by HuR, and dCK is related to gemcitabine response; the easy conclusion is that HuR is related to gemcitabine response. However, a lot of other proteins (e.g., Wee1) are also effected by HuR and may impact tumor aggression and patient survival [43]. It cannot be ruled out that the impact of gemcitabine on HuR is a greater determinant of patient survival than the impact of HuR on the activity of gemcitabine.

Nonempirical Studies of Drug Response Markers

Determinants of Drug Resistance

Response to chemotherapeutics depends on (i) the cancer cell susceptibility to the agent, (ii) the toleration of the agent by the patient's other cells, (iii) the ability of the patient's immune system to respond positively to the action of the chemotherapeutic, and (iv) the availability of the drug at the location of the cancer cells (see Fig. 2).

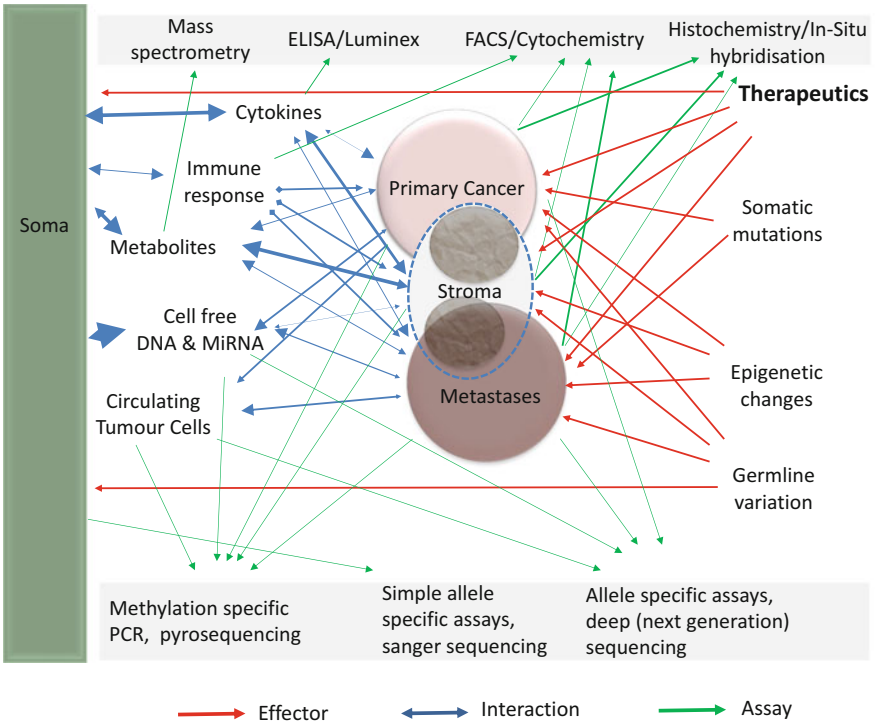

Fig. 2 Sources of biomarkers. The nature of the body (soma), cancer, and stroma is defined by their genetics and epigenetics and also by the actions of external factors such as therapeutics. As a result, the different forms of tissue will release material (cells, exosomes, DNA/miRNA, metabolites, and cytokines) which in turn may act on the tissues to change them further. A picture of how a patient is likely to benefit from a given therapeutic can be obtained by sampling the cancer and stroma or by assay of the substances released by, and corporeal response to, the cancer

Knowledge of how a drug works allows prediction of how response might differ between individual patients dependent on the nature of their particular tumor or germline genetic background.

Pyrimidine-based chemotherapeutics act in a variety of ways: inhibiting various aspects of nucleotide biosynthesis, incorporating into RNA (preventing cell growth and division) or, via incorporation into DNA, stalling replication forks, thus causing DNA breaks and cell death. These modes of action require processing of the drugs along nucleotide biosynthetic pathways which are represented schematically in Fig. 3. The simplest forms of pyrimidine-based chemotherapeutics are nucleobases, the best known example being 5-FU. Nucleobases can be converted to nucleosides by the addition of a sugar moiety or they can be directly converted to nucleotides by the addition of a phosphorylated sugar[44]. 5-FU is converted to a deoxy-nucleoside (5-fluoro deoxy-uridine, 5FdUrd) by the enzyme thymidylate phosphorylase (TP; otherwise known as platelet derived-endothelial cell growth factor) or to a ribonucleoside (5-fluoro uridine, 5-FUrd) by uridine phosphorylase (UP). Conversion of uracil directly to a nucleotide is catalyzed by orotate phosphoribosyltransferase (OPRT) which occurs in the gastrointestinal tract, and inhibition of OPRT by potassium oxonate reduces the toxicity of 5-FU in the gastrointestinal mucosa [45]. Alternatively, the orotate ring of 5-FU can be reduced by the enzyme dihydropyrimidine dehydrogenase (DPD) to the less toxic nonaromatic compound 5-fluorodihydrouracil, which in turn is converted by dihydropyrimidinase and β-ureidopropionase to the effectively nontoxic compound α-fluoro-β-alanine [46].

Nucleosides are phosphorylated in cells to first give nucleoside monophosphates. For gemcitabine, this is carried out by cytidine kinase, for dFdUrd by thymidine kinase, and for 5-FUrd by uridine kinase. Monophosphate deoxynucleotides derived from gemcitabine and 5-FU both inhibit the enzyme thymidylate synthetase (TS) which catalyzes the transfer of a methyl group from folate to uracil to produce thymidine monophosphate. Inhibition of TS will therefore reduce the nucleotide pool and arrest the cell cycle. This explains the sensitivity of therapies (in particular 5-FU) to folate levels. For this reason, the reduced folate analogue leucovorin is usually added to 5-FU, both in traditional 5FU monotherapy and in novel combination regimens such as FOLFIRINOX. Leucovorin not only has the advantage of not requiring dihydrofolate reductase (DHFR) but also appears to induce increased DHFR activity [47]. The ribonucleotide produced from 5-FUrd by uridine kinase or by OPRT is converted to the diphosphate nucleotide by uridylate kinase. Both the ribonucleotide and deoxyribonucleotide diphosphates are converted to triphosphate nucleotides by nucleoside diphosphate kinases such as NME1 (NM23-H1); this would allow incorporation of 5-FU derivatives into RNA inhibiting transcription and translation or causing DNA breaks. Knockdown of NME1 increases sensitivity to 5-FU [48] but increases resistance to other agents such as cisplatin [49], suggesting the inhibition of TS by the diphospho-deoxynucleotide is the critical element of 5-FU toxicity, at least in cell lines.

Nucleoside monophosphate kinase (NMK) catalyzes the conversion of monophosphate to diphosphate nucleotides; on the gemcitabine pathway, this will give difluorodeoxy cytidine diphosphate (dFdCDP), a potent inhibitor of the enzyme

ribonucleotide reductase, which is comprised of the subunit RRM1 and either RRM2 or its stress protein homologue p53R2. Ribonucleotide reductase converts ribonucleotides to the deoxynucleotides necessary for DNA synthesis, so inhibiting its activity will prevent cell division.

Capecitabine (Xeloda) is often described simply as an orally available 5-FU prodrug. Processing of capecitabine almost certainly begins in the liver, where an aliphatic chain is cleaved from the amine group on the orotate ring by the enzyme carboxylesterase to give 5-deoxy-5-fluorocytidine (DFCR). DFCR then needs to be converted into 5-deoxy-5-fluorouridine (DFUR) by cytidine deaminase (CDD) before being converted into 5-FU by thymidine phosphorylase (TP). It is not so clear where these latter two steps occur; it is quite possible that liver CDD and TP carry out these processes, but it is equally possible that this occurs within cancer cells and stromal cells or even extracellularly. Certainly overexpression of CDD in cancer cells increases sensitivity of these cells to DFCR [50]. In contrast, gemcitabine deamination by CDD reduces its toxicity.

As shown in Fig. 3, there are a large number of intermediates lying between prodrugs and their effectors, and the intermediates will interact in a complex way. For example, inhibition of TS by monophosphate nucleotides or ribonucleotide reductase by diphosphate nucleotides will reduce DNA synthesis and so reduce the toxicity of triphosphate nucleotides. It is not only the absolute concentration of these intermediates in a cancer cell that determines the effectiveness of the drug but also the relative concentrations. Concentration will be effected by rate of metabolism inside and outside of cancer cells and also by rate of cellular import and export. Nucleosides and to a lesser extent nucleobases are transported into cells by concentrative (cNTs) and equilibrative (eNTs) nucleoside transporters and are transported out by eNTs. Nucleotides are not transported by nucleoside transporters, and so the phosphorylation of nucleosides will trap metabolites in cells (or prevent their entry), while adding a ribose sugar to a nucleobase will increase import (or export).

Genetic variation in a cancer cell can clearly alter that cell ability to metabolize cytotoxic agents, and it has been demonstrated that genetic heterogeneity does indeed correlate with the variability of pancreatic cancer cell lines to chemotherapeutics [51]. Cancer is a disease characterized by somatic mutations. The genomes of cancer cells are therefore different to the patient's germline. As the objective of chemotherapy is to kill the cancer and not the patient, this offers an opportunity if the chemotherapeutic can be targeted so as to exploit this genetic difference; the genetic difference would then be the most obvious response marker. However, most chemotherapeutics are aimed more generically at the functional differences between cancer cells and their hosts (such as increased cell division). Furthermore, detecting the specific somatic mutations that define sensitivity may be difficult if the tumor load is at a low (treatable) level. For example, following resection of a primary tumor, it is to be hoped that the bulk of the cancer cells have been removed; the remaining cells are now the problem, and these may be genetically very different from the tumor sent to the pathology department.

Germline variants will be carried by cancer and noncancer cells, potentially defining the rate of activation or clearance of chemotherapeutics as well as the

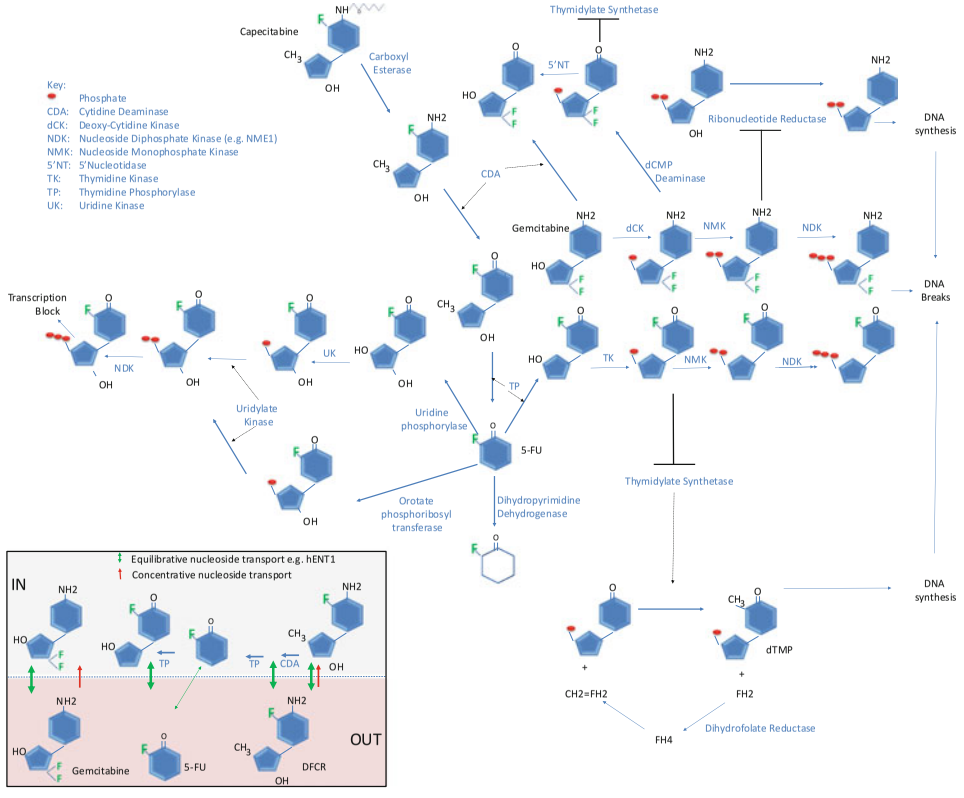

Fig. 3 Schematic of pyrimidine metabolism showing 5-FU, gemcitabine, and capecitabine. Structural features are only shown where they differentiate one form of ribose or base from another (e.g., 3′ hydroxyl groups and orotate ring nitrogens are not shown as they are uniformly present). Gemcitabine would be as for cytidine (an amino-substituted nucleoside) with two fluoro residues on the 2′ carbon of the ribose. 5-FU is uracil with a fluoro residue on the fifth carbon of the

toxicity of the drug and effecting tolerance of the agent and effectiveness against the cancer cells.

Germline Determinants of Benefit: Pharmacogenomics

Dissection of the pathways involved in drug metabolism seems a good place to start when looking for a biomarker. An individual's enhanced ability to clear a drug may reduce toxicity of the compound. On the other hand, too efficient clearance may cause low concentrations of the active compound and result in no antitumoral effects.

Germline polymorphisms of the genes encoding OPRT, DPD, cytosine deaminase (CDA), 5′-nucleotidase (5'NT), uridine monophosphate kinase (UMPK), TS, and methylene tetrahydrofolate reductase (MTHFR) have all been associated with response to pyrimidine-based chemotherapeutics [44, 52, 53]. Neutropenia following treatment with gemcitabine occurs more frequently in patients with a particular haplotype of SNPs within CDA [54]. On the other hand, the same SNPs have been reported to give better survival in patients with acute myeloid leukemia treated with AraC [55], perhaps reflecting the balance between increased sensitivity of tumor cells and reduced tolerance of the rest of the soma. The c.A79C polymorphism of CDA translates into p.K27Q. Individuals with the A/A genotype have a lower CDA activity [56], and in a meta-analysis of seven independent studies the polymorphism did associate with gemcitabine-related severe anemia. However, there was no significant relation with response rates (at least in patients with non-small cell lung cancer) [57].

Systemic 5-FU is mostly detoxified by DPD activity in the liver. Severe and occasionally life-threatening toxicity following administration of 5-FU is commonly associated with DPD deficiency which in turn is associated with the genotype of the DPD gene (*DPYD*) [58]. Two individual polymorphisms – *DPYD**2A (IVS14 +1G>A, c.1905+1G>A, or rs3918290) and c.2846A>T (p.D949V or rs67376798) have consistently shown connection with DPD deficiency [59], and screening for the DPYD*2A variant has been be used for prospective dose adjustment in the clinical routine setting [60]. A meta-analysis examining the relevance of the c.1679T>G, c.1236G>A/HapB3 and c.1601G>A polymorphisms showed that the first two were associated with severe toxicity to 5-FU [61].

Individual SNPs in nucleoside transporters have yet to be shown to have any effect on sensitivity to pyrimidine-based drugs, but a haplotype based on three SNPs

Fig. 3 (continued) ring. Capecitabine is a nucleoside lacking a hydroxyl at the 5′ carbon of the ribose and having an aliphatic chain on the amine group of the base. Like 5-FU, capecitabine has a fluoro residue on the 5′ carbon. Tegafur (the 5-FU prodrug in S1) is not shown in the diagram, but like capecitabine it has a reduced ribose moiety that needs to be cleaved off (giving 5-FU) in order for the drug to become active. Inset is potential transport mechanisms in the tumor: In and out of the cells (stromal or cancer cells). In this figure it is assumed that 5-deoxy-5-fluorocytidine (DFCR) is produced in the liver from capecitabine and converted to 5-deoxy-5-fluorouridine in cancer or stromal cells

in the promoter region of hENT1 (SLC29A11345C4G, 1050-G4A, and 706G4C) has been shown to associate with higher median expression of hENT1 in a Cos-1 cell luciferase reporter assay [62]. This reporter assay does not mean that hENT1 transcription levels will be higher in patients, nor does it mean that this will specifically relate to cancer cell transcription, nor that transcription rate will equate to protein levels in the tumor, it does give one mechanism for heterogeneity in patients. Similarly, common variants in the dCK promoter region have been shown to give up to fourfold differences in transcription level [63]. Again this may not lead to any difference in a given tumor cell's sensitivity to gemcitabine, but it does indicate one way in which patient heterogeneity can be defined that is relevant to drug metabolism.

The principle enzymatic target of gemcitabine is ribonucleotide reductase, which is a protein complex of the RRM1 and RRM2/p53R2 proteins. In non-small cell lung cancer, a haplotype of SNPs in RRM1 was found to associate with survival, although no correlation with protein level was observed [64].

Gemcitabine also causes replication fork stalling and hence DNA damage as an alternative mechanism of toxicity, associating it with the broad range of other DNA damaging chemotherapeutics. The capacity to repair DNA is obviously influential in determining response to an agent that causes DNA damage. Polymorphisms in *ATM* and *CHK1* genes have been associated with overall survival in patients treated with gemcitabine [65]. ATM is involved in detecting DNA breaks and CHK1 acts downstream of ATM to arrest the cell cycle and promote DNA repair.

Cyclin D1

The minor allele of the c.G870A SNP (A) in Cyclin D1 gives an increase in a splice variant form of Cyclin D1 (Cyclin D1b) which results in a truncated protein with gain and loss of function characteristics. The minor allele frequency is approximately 40% in Caucasian populations, and in a mixed population of 300 German patients undergoing resection for PDAC, the 50 patients homozygous for the minor allele had poorer survival (quoted as 15.1 months) than heterozygotes (quoted as 21.5 months) or homozygote major allele (quoted as 29.4 months). The authors also state that in multivariate Cox regression analysis, "a moderate/strong expression of the Cyclin D1 protein was identified as [an] independent prognostic factor for poor outcome with a relative risk of 1.82 (95%CI [1.28–2.67]; $P = 0.003$)" [66].

This is clearly an evidence for a prognostic significance (albeit weak evidence), but lack of chemotherapy data makes this of little value in relation to treatment. However, there is increasing evidence for a relationship between Cyclin D1b and response/prognosis in other cancers, including greater risk of recurrence of breast cancer in patients with D1b. In some studies, this is reported to be independent of D1a protein isoform [67], while in others it is in combination with D1a [68]. Most patients in these analyses had combined doxorubicin and cyclophosphamide treatment, and mouse model data suggests D1b is associated with PARP1 activation (and so DNA damage response) [69]. It is possible that increased D1b at once makes cells more susceptible to spontaneous mutation and more able to survive and proliferate with DNA damage: hence more resistant to DNA damaging agents. D1b is also

associated with anchorage-independent cell growth [70] and is a potential target in its own right for novel forms of therapy.

However, all of the above interpretation has to be treated with caution given findings with prostate cancer. In benign prostate, c.A870 appears to be associated with the level of D1b (as would be expected), but there is no such association in prostate cancer. This seems to be due to the upregulation of the splicing factor ASF/SF2 in prostate cancer and the preferential binding of this protein to the c.G870 transcript [71].

Immune Response

Pancreatic cancer is by nature immunosuppressive, but the level of this suppression is tumor and patient specific with patients showing better immune reactions having the best survival [72, 73]. Measurement of immune response could be a marker of how much a patient will benefit from therapies that co-opt the immune system to fight the cancer. Neutrophil-to-lymphocyte ratio (NLR) is a general prognostic marker; the level of change after gemcitabine-based chemotherapy may also relate to response to the therapy [73].

In an adoptive immunotherapy study where effector cells were selected and expanded using zoledronate, it was observed that the response was better in patients with a high baseline level of lymphocytes [74]. This is of course a special case in terms of a therapeutic biomarker, given that each individual patient was both the source and beneficiary of their treatment.

Evaluation of an immune response to peptide or protein cancer vaccines has largely been disappointing to date; although it has proved possible to engender a specific immune response with the vaccines, this has not resulted in a clear survival benefit [75]. However, whole-cell vaccine approaches, although far from a panacea for PDAC, have been reported to engender an immune response that relates to survival, for example, GVAX (tumor cells engineered to secrete GM-CSF) increases thyroglobulin antibodies, and the level of induction has been claimed to relate to survival [76]. More generally whole-cell vaccines including algenpantucel (allogeneic pancreatic cancer cells engineered to express murine galactosyl transferase) give variable elevation of anti-mesothelin and/or anti-calreticulin antibodies which also correlate with survival [77].

Calreticulin surface exposure will cause macrophage phagocytosis of cancer cells and is a potent inducer of immunogenic cell death (ICD). Its exposure is therefore defined as a key damage-associated molecular pattern (DAMP) quantifying ICD [78]. The ability to cause ICD may be a crucial determinant of whether chemotherapy will be effective or not. CD47 is believed to inhibit exposure of calreticulin [79], and CD47 upregulation causes resistance to agents such as sorafenib [80]. Conversely, loss of CD47 can cause increased immune clearance, at least in human papillomavirus (HPV) carrying oropharyngeal squamous cell carcinoma.

ER stress-induced autophagy (which can be induced by some forms of chemotherapy) reduces exposure of calreticulin [81]. On the other hand, chemotherapy-induced autophagy can also facilitate ATP secretion, which stimulates ICD [82], and

it has been shown that cells lacking the autophagy protein LAMP2A do not expose calreticulin in response to the chemotherapeutic mitoxantrone [83].

Selected Proteins Associated with Drug Metabolism, Transport, or Repair in Tumors

Examination of Figs. 2 and 3 gives an idea of how complex and tangled the pathways are that determine response. Nevertheless, picking the level of a specific protein to measure on the assumption that it might be the key element is by far the most popular approach to assess potential response. Typically, proteins are quantified in a resected tumor sample, despite the fact that the cancer cells resected from a patient are not themselves the target for subsequent therapy and that protein levels are determined at least in part by the tumor microenvironment, which is likely to be very different in metastatic deposits than in the primary.

Enzymes Involved in Drug Metabolism

The levels and activities of proteins involved in drug metabolism are modulated by many factors other than the primary sequence of the genes encoding them. Analysis of epigenetic changes to the genes is one approach, but direct quantification of the proteins in drug pathways is a more direct method to investigate a link to response or even better measure the protein's activity. The challenge is to identify a relevant and accessible clinical sample to analyze. A germline genetic change may only have an impact in one cell type (e.g., cancer, stromal, or liver) but can be measured in any sample from the patient. A somatic mutation is likely to be specific for the cancer cell but can be identified wherever cancer DNA is found (e.g., biopsy, circulating tumor cells or cell-free DNA). High protein levels of a drug-metabolizing enzyme may link to improved response (e.g., increased cancer cell death) when identified in a cancer cell or poor response (e.g., reduced tolerance) when seen in a hepatocyte, and so context is all important.

The plasma levels of a metabolite following the administration of a standard "test dose," enzymatic activity in peripheral white blood cells, or analysis of enzyme expression and/or activity in liver biopsies can all be used as measures but will not necessarily inform about critical features of drug metabolism in the tumor. An individual may be a "slow metabolizer" systemically, whereas the tumor itself may express high levels of the metabolizing enzyme and so clear the tumor microenvironment. As seen in Fig. 3, gemcitabine is metabolized away from its toxicity pathway by CDA, while capecitabine is pushed through its toxicity pathway by the same enzyme. The effect of CDA on 5FU is somewhat more complex; clearly it will change the flux of 5-FU metabolism, but depending on cellular environment, this could increase toxicity (removing non-fluorinated orotate moieties so increasing flux in the direction of toxic nucleotide metabolites) or decrease toxicity (removing fluorinated nucleosides).

Ciccolini et al. measured serum CDA activity in cancer patients treated with gemcitabine, 64 given monotherapy (of whom 40 had pancreatic cancer) and 66 given combination therapy (of whom 12 had pancreatic cancer): patients with higher CDA activity had less treatment-related toxicity [84], consistent with

previous case studies linking low CDA activity to hematologic toxicity [85]. The same group later reported data on serum CDA from 40 patients with advanced pancreatic cancer; 23 had received gemcitabine monotherapy and the rest combination therapy. Response was measured using RECIST, and the authors claimed significantly higher CDA activity in the 11 patients with progressive disease compared to patients with stable disease or a partial response [86]. In direct contrast, the group reported that an adrenocortical carcinoma patient with severe capecitabine-associated toxicities had high CDA, consistent with the contrasting roles of CDA in gemcitabine and capecitabine metabolism (Fig. 3).

Some studies indicate that the intratumoral expression of DPD, as measured by immunohistochemistry (protein level) or in situ hybridization (RNA level), may be inversely linked to survival in patients treated with 5-FU [87] and its associated prodrugs (capecitabine [88] or tegafur [89]) as would be expected as DPD in the tumor will detoxify the drug. More difficult to explain, DPD has also been associated with improved survival in patients treated with gemcitabine [90], raising issues as to whether low DPD in the tumor is a prognostic or predictive biomarker. To add further complexity to this somewhat confused story, although a high ratio of TP to DPD has been reported to be associated with good response to capecitabine in rectal cancer [91] and in a pancreatic cancer study [92] (potentially due to high TP rather than low DPD), another study in pancreatic cancer showed the absolute opposite, i.e., improved survival with lower TP/DPD ratio [93]. This illustrates the problem of assuming a mechanism to explain response. Nevertheless, the concept of DPD inhibition as an adjunct to treatment with 5-FU-based prodrugs has been taken forward with apparent success. The DPD inhibitor gimeracil is used along with tegafur in the combination therapy S1, giving higher concentration of 5-fluorouracil in blood and tumor tissue [2], with a reported increased therapeutic benefit.

As shown in Fig. 3, the effect on drug response of the level of nucleoside transporters is difficult to predict. Concentrative nucleoside transporters would be predicted to increase the concentration of potentially toxic metabolites in cancer cells but will also pump unsubstituted nucleosides into cells changing the flux of metabolites after entry into the cell and determining the consequence of treatment with prodrugs. Equilibrative nucleoside transporters will pump gemcitabine, 5-FU, and other toxic compounds into cancer cells but also out of cells. Numerous cell line studies have examined the effect of different levels of nucleoside transporters on response to gemcitabine and 5-FU with contradictory results [94]. In contrast, studies in patients have fairly consistently shown that high levels of hENT1 are associated with better prognosis in PDAC patients treated with adjuvant gemcitabine [95]. Of importance this does not seem to be true in patients treated with 5-FU [3]. One study indicated that hENT1 levels were not prognostic in patients with advanced pancreatic cancer treated with gemcitabine [96], although this is possibly because of the choice of antibody used to analyze the hENT1 levels [97].

Drug Targets

The discussion above indicates how difficult it is to predict how the levels of enzymes controlling the flux of toxic metabolites will impact on response.

Superficially, it would seem to be much easier to predict the effect of an increased level of a drug target. Obviously, this will only apply assuming no other factor is limiting: the level of a target is immaterial if the drug cannot reach it in an active form. However, considering the target in isolation, it is very tempting to assume that the greater the amount of target in the tumor, the greater the amount of drug required and so the lower the expected level of response. A problem with this is that cancers may become more resistant by losing dependence on a drug target, in which case a tumor might have very low levels of a redundant target protein and be more resistant than a tumor which has very high levels of an essential target protein. Another problem is the potential for multiple targets for a single drug, or the use of drugs with different targets in combination, for example, inhibition of target A may reduce an effect on target B, and so low levels of target A may result in resistance because target B is protected.

It can be seen in Fig. 3 that pyrimidine-based chemotherapeutics have multiple targets. A number of groups have looked at tumor levels of ribonucleotide reductase subunits and TS as possible response markers. Increasing resistance during treatment of cell lines with gemcitabine seems to be associated with increasing levels of RRM1 and RRM2 [98], and reducing the level of RRM1 restores sensitivity to resistant cell lines [39]. Consistent with this, some groups have shown that low RRM1 measured in tumors at the protein level [99] or at the RNA level [100] is related to good prognosis in patients treated with gemcitabine. However, other groups have dismissed this association, again at both the protein [101] and transcript level [102–104]. It is also telling that particular p53 mutations which increase gemcitabine sensitivity, apparently by increasing dCK, also increase RRM1 and RRM2 [105], so higher (rather than lower) levels of ribonucleotide reductase would be a passenger of this particular genetic mechanism for defining sensitivity.

It has proved harder to demonstrate that high TS is associated with poor response to 5-FU, partly because cell lines tend to be sensitive to modulation of TS level regardless of 5-FU treatment and partly because 5-FU effectively induces TS expression in most cell lines. However, by expressing TS using a Tet-OFF system in a colorectal cell line, it has been possible to confirm (at least in this system) that there was a linear relationship between TS and 5-FU sensitivity [106]. In patients (as in cell lines), TS is induced by 5-FU and, at least in lung cancer, this increase is associated with acquired resistance to 5-FU [107].

In patients, the relationship between TS and survival is even more confusing than that seen between ribonucleotide reductase and survival. In some studies with pancreatic cancer, low TS is associated with good response [108]; in others high TS is associated with good response to 5-FU [109] and gemcitabine [110] even though this is otherwise associated with poor prognosis. In still further studies, low TS is linked to good prognosis in pancreatic cancer patients without treatment [111]. This contrasts with studies suggesting high TS is associated with good survival, but this is not related to 5-FU [112]. This confusion is not unique to pancreatic cancer; in colorectal cancer, high [113] and low [114] TS have also both been associated with good response to 5-FU.

It is of course very possible that the differences seen between studies looking at TS and ribonucleotide reductase are due to differences in patient groups and/or analytical methods (the antibody used or method to measure transcripts). Alternatively, this could be explained by a statistical anomaly. It is so manifestly obvious that the level of the drug target in a cancer cell should be relevant to response (confirmed by manipulation of their levels in cell lines) that anyone interested in this area is likely to test this out. Failure to show a relationship will of course be quickly forgotten, while chance demonstration of an association will be published.

This is not to say that drug targets are not excellent candidates as response biomarkers; the problem is that with traditional chemotherapeutics, there are usually multiple potential targets. The current progress in targeted therapy (see Table 1) provides a safer basis for use of targets as biomarkers. Certainly absence of targets within cancers is likely to mean the patient will not respond to the therapy.

Secreted Protein Acidic and Rich in Cysteine (SPARC) Stroma and Nab-Paclitaxel

To improve solubility of taxanes, paclitaxel was bound onto albumin nanoparticles to form nab-paclitaxel (abraxane). SPARC (osteonectin) is an albumin-binding protein which plays a key role is deposition of extracellular matrix. It is expressed on cancer cells and was confirmed to increase intracellular accumulation of nab-paclitaxel [116]. Although initial data seemed to support high SPARC as a marker for response to nab-paclitaxel in various forms of cancer [117], data from the MPACT trial cast doubt on this [118]. This negative finding is consistent with observations in breast cancer where no relationship with survival has been reported with tissue or serum SPARC in patients treated with abraxane [119].

Table 1 Targeted therapy

Target	Drugs in development
EGFR	Erlotinib/SKLB261
IGF-1R	AMG479
JAK/STAT	Ruxolitinib
AKT	RX-0201
MEK	Trametinib/AZD6244
PI3K	BKM120
Wnt	OMP-54F28/LGK974/vantictumab omp-18RS
mTOR	Everolimus/metformin
VEGFR	Sorafenib/axitinib/foretinib/nintedanib
VEGF	Bevacizumab
PARP	Veliparib/olaparib
NOTCH	OMP-59R5
SMO	Vismodegib
TGFβR1	LY2157299

Adapted from Karanikas et al. [115]

Proteins Involved in DNA Repair

PARP compensates for loss of BRCA2; therefore, PARP inhibitors, such as olaparib, are more effective in patients with BRCA2 mutations [120]. BRCA2 itself is relatively rarely mutated in pancreatic cancer, but a much larger group of patients have a pattern of mutation that gives a deficiency in recombination repair analogous to loss of BRCA2 (so called BRCAness), and these patients may benefit from drugs like olaparib which inhibits PARP [16].

As described above, ATM is involved in the cell's response to DNA double-strand breaks, and elevated levels of ATM have been identified in premalignant and invasive pancreatic tumors [121]. Low ATM (with normal Tp53) was found to be associated with poor prognosis in pancreatic cancer [122]. Of the 396 patients in this study, the majority had some form of chemotherapy, but critically 21 had neo-adjuvant therapy only one of whom had loss of ATM, and this patient showed no objective sign of response.

Tp53

Because p53 is mutated in approximately half of all PDAC and is such a crucial gene in the response to DNA damage and other forms of stress caused by chemotherapeutics, mutations in Tp53 are an obvious place to look in regard to drug response. Indeed agents that restore wild-type p53 function do seem to sensitize cancer cell lines to chemotherapeutics such as adriamycin and gemcitabine [123]. It may well be that specific forms of Tp53 mutation do relate to response, but to date attempts to associate the histological p53 levels (mutant p53 being more stable than wild type) or mutant p53 sequences to response have proved unsatisfactory.

Combinations of Protein Markers

It may not be the absolute level of a protein but rather its level relative to other proteins that affects response. For example, 5-FU is processed toward its toxic metabolites by TP and is cleared by DPD, so it is reasonable to assume that a high TP-DPD ratio would relate to efficacy of 5-FU and there is some evidence for this [92]. Interestingly, hENT1-DPD ratio relates to efficacy of capecitabine [92], whereas gemcitabine is affected by hENT1 levels but 5-FU is not, so this suggests that the agent does not enter cancer cells in the form of 5-FU.

Proteins Indicating Epithelial to Mesenchymal Transition (EMT)

The intrinsic capability of cancer cells to transition from an epithelial phenotype, with cobblestone appearance, to a mesenchymal phenotype, with a fibroblastic, spindle-like appearance, has been linked to drug resistance [124]. Exposure of pancreatic cell lines to cytostatic drugs, such as gemcitabine, results in activation of the EMT pathway [125], and cells with high levels of the epithelial marker, E-cadherin, combined with low expression of its transcriptional repressor, Zeb-1, have been shown to be sensitive to three commonly used chemotherapy drugs gemcitabine, 5-FU, and cisplatin[126]. A phase II clinical trial combining MEK1/2 inhibitor, selumetinib, with the EGFR

inhibitor, erlotinib, in patients with advanced PDAC showed that patients with tumors expressing higher levels of E-cadherin, i.e., epithelial phenotypic cells, were significantly associated with treatment sensitivity [26]. Several molecular pathways have been linked with EMT. Embryonic signaling pathways involved in cell differentiation, Hedgehog, Wnt, and Notch can be reactivated in response to gemcitabine treatment [127], and increased expression of these molecules may result in inactivation of the apoptotic pathway or increased expression of drug efflux pumps thereby resulting in the lack of cell sensitivity to cytostatic agents. Additionally, some epithelial cell types are known to be addicted to K-Ras mutations; however, upon transition to mesenchymal cell type, dependency is overcome. This loss of the K-Ras dependency gene signature has been shown to result in a loss of sensitivity to EGFR kinase inhibitors [128]. Therefore, analysis of combinations of markers that indicate the level of mesenchymal or epithelial cancer cells may prove effective in predicting drug response.

Stroma

There is little doubt that stroma is a major determinant of response, but the nature of this involvement is far from clear. Early work with transgenic animal models strongly suggested that stroma prevents chemotherapeutics such as gemcitabine reaching cancer cells, therefore determining level of response [129]. Hedgehog pathway inhibition improved delivery of gemcitabine in a mouse model (presumably by reducing stroma), but the smoothened inhibitor vismodegib did not improve response to gemcitabine in early phase trials [130]. Subsequent work indicated that far from promoting cancer development and spread, stroma could restrain the cancer: depletion of stroma with sonic hedgehog inhibitor was shown to accelerate PDAC progression [131], furthermore eliminating fibroblasts from a transgenic mouse model increased immunosuppression and again accelerated progression [132]. It appears that it is not the level of stroma so much as the type of stroma that matters, for example (again in a mouse model), small metastatic lesions with little stroma seem as resistant (or sensitive) as larger metastatic lesions with high levels of stroma to a combination of gemcitabine and abraxane [133]. At least part of the explanation may lie with stromal remodeling by tumor expressed focal adhesion kinase (FAK); the FAK1 protein seems to reduce CD8 lymphocyte invasion of the tumor microenvironment and increase the number of immunosuppressive cells [134], as a consequence making cancers more resistant to immuno- and chemotherapy [134]. FAK1 may therefore prove to be an important response marker, even if FAK1 inhibition is not successful as an adjunct to other therapies.

Another possibility is that high interstitial pressure may prevent drugs reaching their target because of reduced perfusion and compression of intratumoral vessels. A factor in this is the glycosaminoglycan polymer hyaluronan (HA) that accumulates in stroma. Drugs targeting HA (such as PEGPH20) could therefore increase efficacy of chemotherapeutics. There are early indications that this can be effective if there is high levels of HA [135]

Conclusion

The complexity and interrelatedness of the factors determining drug response means that complex and multifactorial biomarker approaches are needed. The real challenge is to get markers into the clinic; failure of oversimplistic single markers or over-fitted panels of markers is to be expected and should not discourage continued work on rigorously validating new leads. That validation will require careful choice and application of endpoints; in this respect, expectations probably need to be managed, biomarkers predicting cure will certainly be easy to validate, but biomarkers predicting small incremental improvements are at once more probable and less easy to test.

Cross-References

- ▶ Adjuvant Chemoradiation Therapy for Pancreatic Cancer
- ▶ Adjuvant Chemotherapy in Pancreatic Cancer
- ▶ Approaching Pancreatic Cancer Phenotypes via Metabolomics
- ▶ Chemotherapy for Advanced Pancreatic Cancer
- ▶ Differential Therapy Based on Tumor Heterogeneity in Pancreatic Cancer
- ▶ Emerging Therapeutic Targets in Pancreatic Adenocarcinoma
- ▶ Epigenetic Pharmacology
- ▶ Neoadjuvant Chemotherapy in Pancreatic Cancer
- ▶ Precision Medicine Based on Next-Generation Sequencing and Master Controllers
- ▶ Stromal Inflammation in Pancreatic Cancer: Mechanisms and Translational Applications
- ▶ Vaccine Therapy and Immunotherapy for Pancreatic Cancer

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Approaching Pancreatic Cancer Phenotypes via Metabolomics

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Abstract

Metabolomics, one of the latest omics' technologies, focuses on the global, quantitative, and simultaneous measurement of endogenous metabolites in a biological sample. Investigation of either individual metabolites, a panel of metabolites, or a broad metabolite profile (metabolome) can be carried out in cells, tissues, or body fluids. Recent publications indicate that there is an enormous, constantly growing multitude of metabolomics applications in oncology. As a translational research tool, metabolomics provides a link between basic *in vitro* laboratory data to *in vivo* preclinical results and clinical oncology and enables systems biology insights. In the present chapter, the current and potential future applications of metabolomics in PDAC research are focused on the clinical aspects of diagnostics.

Keywords

Metabolomics · Metabolite profiling · Mass spectrometry · Nuclear magnetic resonance · Metabolism · Biomarker · Systems biology approach · Stable isotope-labeled metabolites · Metabolite flux · MS-based metabolite imaging

Introduction

Metabolomics, also referred to as metabolite profiling, metabonomics, metabolic fingerprinting, or metabolic phenotyping, is defined as a comprehensive, simultaneous, and (semi)quantitative measurement of endogenous metabolites within a biological system [1–3]. It represents a modern omics' technology applying automated analytical instrumentations to facilitate the assessment of many different metabolites within the context of alterations in gene regulation or altered kinetic activity of enzymes, and thus changes in metabolic reactions [2]. Therefore, metabolomics complements upstream biochemical information obtained from genes, transcripts, and proteins, thus widening the current understanding of cell biology, physiology, and medicine by linking cellular pathways to biological mechanisms.

Cellular processes and the physiological status are most closely reflected by the patterns of metabolites (metabolome or metabolite profile), the small molecular weight (<1.5 kDa) endogenous and exogenous molecules such as nucleotides, carbohydrates, amino acids, lipids, hormones, cofactors, and vitamins whose levels are highly responsive to both genetic and environmental factors. Many of these metabolites represent building blocks of the genome, transcriptome, proteome, and cellular membranes or are used as signaling molecules or energy sources. The precise number of human metabolites (the size of the human metabolome) is unknown, with estimates ranging from thousands to tens of thousands.

Compared to other omics' technologies, metabolomics reflects the endpoint of the omics' cascades [4] and the closest snapshot of the cellular phenotype (Fig. 1). In contrast to regulated genes or enzymes, metabolites represent the functional status of the organism. This deep insight into the actual phenotype of any biological system is

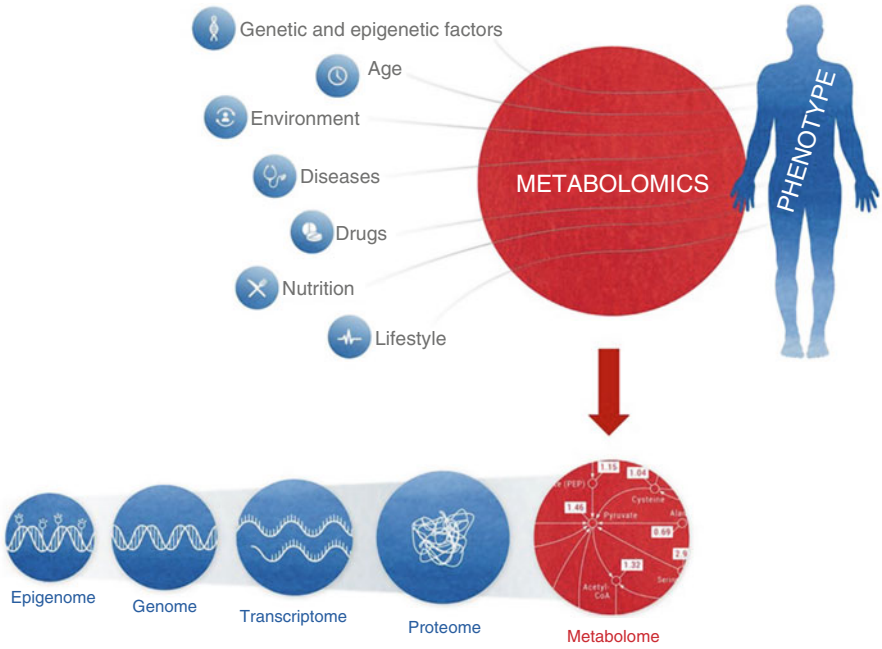

Fig. 1 Metabolomics provides a direct, integrated characterization most closely related to the phenotype

metabolomics' advantage over other omics' technologies. Although, other features of metabolomics are similar to those of genomics, transcriptomics, or proteomics, including the ability to measure both *in vitro* and *in vivo* samples such as cells, tissues, and body fluids. Up-to-date metabolomics represents the ideal approach to understand the current status of a cell or organism of interest and how it is affected by disease, drug treatment, nutritional status, lifestyle, or environment. The resulting characteristic fingerprints can serve as metabolic biomarkers. Metabolic biomarkers are translational across species based on highly conserved biochemical processes and molecular structures, while sequence-based biomarkers (e.g., genes or transcripts) vary between biological classes. This makes them especially useful in the transition from preclinical to clinical studies.

Together with the application of sophisticated statistical approaches, the vast amount of metabolomics data generated from instrumentation can be analyzed and mined, thereby aiding biological and biochemical interpretation (Fig. 2). The success of metabolomics studies is highly influenced by the quality of the investigated sample, innovative instrumentations, sophisticated bioinformatics, as well as biological data interpretation to extract the most relevant findings [5]. Consequently, metabolomics data can be used to build databases that can be integrated with pathway maps, or it can be integrated with other omics' data such as genomics and proteomics providing an enhanced holistic understanding of the biological system.

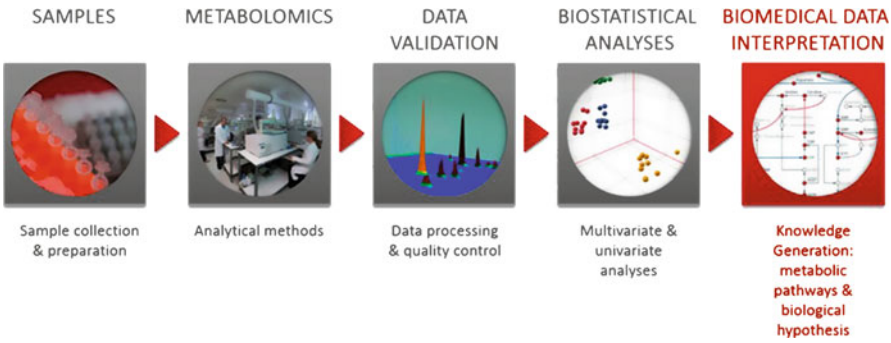

Fig. 2 Experimental setup for metabolomics approach analysis

In this review, general concepts and technical approaches to metabolomics methodology will be highlighted and discussed how it is being applied in the field of pancreatic ductal adenocarcinoma (PDAC) with particular attention to its clinical applications.

Metabolomics: Basic Concept and Insight into Technology

The measurement of single metabolites as a source of information related to health and disease has a long history that precedes the introduction of metabolomics. The Ancient Chinese used ants for the evaluation of urine of patients to detect whether the urine contained high levels of glucose, indicative for a disease now known as diabetes [6]. In the Middle Ages, "urine charts" were used to link the colors, tastes, and smells of urine to various medical conditions, which are metabolic in origin [7]. Over the last decades, metabolomics has developed at an accelerating speed as indicated by the increasing number of metabolomics publications in scientific journals of any biological research field. This development is mainly achieved by increasingly robust, sensitive, and rapid analytical instrumentations allowing the analysis and quantification of hundreds to thousands of metabolites from any biological system.

Metabolites are characterized by a broad repertoire of physiobiochemical properties such as polarity, concentration, structure, mass, and volatility making it challenging to analyze in parallel many different metabolites and/or metabolite classes in a biological sample. Currently, no single technology provides all of the desired properties at once. So far, the main analytical techniques used for the analysis of the metabolome are nuclear magnetic resonance (NMR) spectroscopy and hyphenated techniques (coupling of a separation technique and an online detection technology) such as gas chromatography (GC), liquid chromatography (LC), capillary electrophoresis (CE) coupled to mass spectrometry (MS), flow injection analysis (FIA), and ion mobility spectrometry (IMS). These platforms are complementary in parts so that comprehensive insights can be obtained by combining them.

NMR-Based Metabonomics

NMR spectroscopy can provide measurements for different types and sizes of both polar and non-polar metabolites through analysis of different spectral windows. This technique is based on the energy absorption and re-emission of the atom nuclei due to variations in an external magnetic field [8]. NMR instruments are highly versatile, and with only minor adaptations, users can achieve spectral information for different nuclei (^{1}H, ^{13}C, ^{15}N, and ^{32}P, among others) in solvent or solid samples and even *in vivo* [9]. The major advantages of NMR include its nonbiased metabolite detection and quantitative nature of the data. Furthermore, NMR represents a rapid high-throughput technology, it is non-invasive, non-destructive, and highly discriminatory which can analyze rather crude samples without extensive sample preprocessing and separation. On the other hand, the major problem of NMR technology is its low sensitivity, which limits the majority of currently available instruments to the measurement of approximately 100 metabolites in a single experiment [10]. Mass spectrometry-based technology is preferred in metabolomics and currently has a number of publications exceeding the number of NMR-based publications.

MS-Based Metabolomics

Mass spectrometry is a powerful analytical technology used to quantify known metabolites and identify unknown metabolites (analytes) in a sample. Its high sensitivity and resolution achieved with separation techniques such as capillary electrophoresis, liquid, or gas chromatography allows for the detection of hundreds to thousands of molecules in a single measurement. The complete process involves the conversion of the extracted metabolites into gaseous ions, with or without fragmentation, which are then resolved through the manipulation of electric or electromagnetic fields by their mass-to-charge ratios (m/z) and relative abundances (intensities of detected ions).

Recent technological advances in separation science, ion sources, and mass analyzers have considerably increased the sensitivity, selectivity, specificity, and speed of metabolite detection by MS. The most common ionization techniques in metabolomics encompass, e.g., electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), electron impact (EI), or matrix-assisted laser desorption/ionization (MALDI). Mass analyzers with different resolving powers have also been used in metabolomics. A broad range of mass analyzers can be used such as quadrupole, ion trap, time of flight (TOF), orbitrap, Fourier transform ion cyclotron resonance (FT-ICR), and sector field spectrometers. Sensitivity as well as specificity of the measurement can be increased, and further fragmentation information can be acquired through various combinations of mass analyzers to isolate and fragment target ions and to analyze/detect the resulting fragments. Some of the most commonly used tandem mass spectrometers include combinations of TOF, quadrupole, orbitrap, ion trap, and Fourier transform ion cyclotron resonance (FT-ICR), e.g., quadrupole time of flight (Q-TOF) and triple quadrupole (QQQ). Selection of a

specific MS platform for metabolomics depends on the goal of the metabolomics projects, throughput, and instrumental costs.

Coupling a separation technique to MS provides an excellent solution for complex mixture analyses and has been extensively used in metabolomics. Hereby, analytical separation of metabolites prior to MS analyses offers several advantages: (1) reduced matrix effects and ionization suppression, (2) separation of isomers, (3) availability of orthogonal data (i.e., retention time) valuable for metabolite annotation, and (4) enhanced accuracy in quantification of individual metabolites. Currently, three predominant separation techniques have been incorporated in MS-based metabolomics, i.e., GC, LC, and CE.

GC-MS-Based Metabolomics

GC-MS investigates volatile, energetically and thermally stable metabolites. Due to their poor volatility, GC-MS is less amenable to large, highly polar metabolites. Chromatographic analyses of these metabolites rely on other chromatographic techniques such as LC and CE. GC-MS is limited to volatile compounds. However, relatively few metabolites meet this requirement in their native state, but the number of analyzed metabolites by GC-MS can be further increased by including a prior derivatization step. However, this derivatization step might introduce variability and produce derivatization artifacts. Nevertheless, the high resolution and reproducibility of the chromatographic separation makes GC-MS an excellent tool for complex metabolite extract analyses especially with respect to the differentiation of stereoisomers with a large linear range, e.g., glucose and galactose or oleic acid and elaidic acid.

LC-MS-Based Metabolomics

LC-MS is highly sensitive, typically at the picogram level, and allows simultaneous analyses of multiple metabolites specifically multiple metabolite identification at low concentrations [4]. The coupling of liquid chromatography (LC) to MS (LC-MS) increases specificity and facilitates metabolite quantitation by reducing sample complexity. LC-MS typically involves comparison of the relative abundances of metabolites in multiple samples without prior identification. After selecting interesting features according to statistical criteria, these features can be characterized based on their mass spectral information (accurate mass, isotopic pattern, and fragmentation pattern) and retention time.

CE-MS-Based Metabolomics

CE-MS offers an alternative approach for analyzing anions, cations, and neutral particles in a single run. CE separates metabolites based on charge and size, and it is particularly suitable for the analysis of highly polar and ionic metabolites which can be analyzed with high resolution and sensitivity. A potential limitation of CE-MS might be the poor reproducibility. Recently, the performances of GC-MS, LC-MS, and CE-MS were compared in quantitative metabolomics, and it was concluded that CE lacked the necessary robustness and was the least suitable platform for analyzing complex biological samples [11]. Overall, CE is less frequently used for metabolomics analyses.

Nontargeted and Targeted MS-Based Metabolomics

Metabolomics approaches are often divided into targeted and untargeted applications. As the name suggests, targeted methods [12] are designed to detect and often quantify rather few but specific metabolites of interest within a sample. This approach has the advantage of maximizing the specificity and the sensitivity of MS methods. Furthermore, targeted approaches usually report absolute concentrations based on calibration with authentic standards. In contrast, untargeted global metabolite profiling aims to maximize coverage of many different metabolites, metabolite classes, and metabolic pathways, often compromising the sensitivity and specificity for any particular metabolite. These metabolomics approaches involve less up-front method development compared to quantitative targeted approaches, but require much more data analysis. Interpretation of the hundreds or thousands of resulting ions can be challenging due to a large number of unknown metabolites (analytes with missing structural identification and, therefore, without the exact metabolite name). Identifying and characterizing the structure of metabolites has become one of the major drawbacks for converting raw spectrometric data into biological knowledge, preventing metabolomics from evolving as fast as the other omics' sciences. Furthermore, expertise to integrate metabolomics data and other systems-wide data is still in its infancy.

Metabolomics Applied to PDAC Research

The Pancreas and Its Role in Metabolism

The pancreas has central key roles in the regulation of macronutrient digestion and hence metabolism and energy homeostasis by releasing various digestive enzymes and pancreatic hormones. Hereby, the pancreas acts as an exocrine and endocrine secretory organ. The vast majority of the pancreas consist of exocrine cells knowing to secrete the pancreatic juice containing digestive enzymes, such as amylase, pancreatic lipase, phospholipase A2, lysophospholipase, cholesterol esterase, and proteases (e.g., trypsin and chymotrypsin), into the ducts. These enzymes support digestion and metabolism of carbohydrates, complex lipids, fatty acids, and proteins. In contrast, pancreatic hormones such as glucagon, insulin, ghrelin, somatostatin, amylin, and C-peptide are released in an endocrine manner into the bloodstream. These hormones act as messengers, affecting cells and tissues in distant parts of the human body, and regulate glucose homeostasis. Due to these two main functions of the pancreas (digestion and metabolism of nutrients) and the nature of the involved molecules, metabolomics provides an extremely valuable tool to study the activities of this organ in more detail. Furthermore, metabolomics offers great potential to evaluate metabolite changes connected to abnormal, dysregulated phenotypic characteristics of PDAC cells. A comprehensive overview of the metabolic deregulations in PDAC is discussed in the chapter "New Directions - Metabolism and Pancreatic Cancer" of the present book and was recently reviewed [13, 14]. Briefly, PDAC cells

are characterized by increased glucose uptake and glycolytic activity, addiction to glutamine metabolism [15], increased protein catabolism via enhanced autophagy, as well as upregulated lipid and cholesterol metabolism.

Challenges in PDAC Research

Metabolomics' advancements rely on robust and reproducible measurements as well as low coefficients of variation, crucial for successful metabolomics approaches. Validated protocols including information on sample requirements and handling for metabolomics analysis have been published previously, with emphasis on proper sample collection [16, 17]. Recently, the first validated assay for a holistic human plasma quality control was developed to ensure reliability of the results and secure the investment of a large-scale metabolomics study [18]. Nevertheless, major clinical advances have not yet materialized even though significant scientific progress has been made in the last decade in understanding the biology and natural history of PDAC. Although PDAC shares some of the characteristics of other solid malignancies (e.g., mutations affecting common signaling pathways, tumor heterogeneity, development of invasive malignancy from precursor lesions, and environmental risk factors), there are also unique obstacles that have made progress against PDAC difficult. These include: (i) diagnosis at a late disease stage because of a lack of specific symptoms or biomarkers to facilitate early diagnosis, (ii) dynamic interaction of the tumor with stromal cells creating dense fibrous tissue around the tumor (desmoplasia) that contributes to therapeutic resistance, and (iii) the low number of patients for whom curative surgery is a feasible option. There has been a tremendous initiative to discover novel biomarkers that may aid in detecting the disease earlier, improving prognosis, and predicting response to available chemotherapy. The number of implicated biomarkers in PDAC is staggering.

Explorative Discovery Approaches of Metabolomics in PDAC R&D

Metabolite profiling has been used for various study questions in the field of PDAC encompassing explorative *in vitro* cell culture applications, as well as preclinical and preliminary clinical aspects including samples from humans and animal model systems. Clinical investigation will be addressed later on in this book chapter. Regarding explorative studies, scientists have used metabolomics in a multitude of applications including investigation of PDAC cell autophagy or drug response/resistance. Daemen et al. stratified human pancreatic ductal adenocarcinoma cell lines into subtypes with distinct sensitivities to metabolic inhibitors [19]. Mass spectrometry-based metabolomics was applied to profile the metabolic differences between gemcitabine-sensitive and gemcitabine-resistant PDAC cells [20]. Grüner et al. utilized an established genetically engineered mouse model of spontaneous PDAC to examine the distribution of the small-molecule inhibitor erlotinib, a tyrosine kinase inhibitor acting on the epidermal growth factor receptor (EGFR) in

the healthy pancreas and PDAC by matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS) technology [21]. In a humanized genetically modified mouse model of PDAC, it has been shown that autophagy's role in tumor development is intrinsically connected to the status of the tumor suppressor p53, highlighting important considerations for the treatment of this malignant disease [22]. Cachexia is reported in the majority of advanced PDAC patients and has been shown to worsen prognosis. While substantial research is currently focused on determining the mechanism behind cachexia development, no precise understanding has yet been described. Thus, an initial metabolomics experiment was performed to investigate the difference in serum metabolite levels in PDAC patients with and without cachexia and to analyze the pattern and intraday variation in metabolite levels [23].

Overview of Clinical Metabolomics Biomarkers of PDAC

In order to highlight the current advances in metabolomics biomarker discovery for PDAC, a comprehensive literature survey of metabolomics biomarker studies was done and is summarized in Table 1. Human PDAC metabolomics studies were compiled based on the following criterion: only human studies with equal to or greater than five subjects, only studies using MS or NMR technology, and only studies including additional univariate statistics instead of multivariate analysis (principal component analysis, orthogonal projection to latent structure-discriminant analysis) only. Upregulated or downregulated metabolites from cancer patients versus controls are listed according to the studies' biomarker selection criteria. If a study did not indicate the directional change in metabolite levels, then N/A was inserted. In addition to the literature survey, a patent database was screened to identify patent applications referring to either NMR or mass spectrometry-based metabolomics technology to identify PDAC-related human biomarker candidates (Table 2).

The majority of PDAC clinical biomarker studies that employed MS- or NMR-based metabolome analysis reported significant alterations in glucose, amino acid, and protein metabolism as well as lipid metabolism. These dysregulated pathways represent the key metabolic features of PDAC, although different types of diseases and external stimuli (i.e., diet) can cause variations in the same metabolites, making it difficult to connect metabolomics data to specific metabolic pathways. For example, increased glucose concentrations in the urine of PDAC patients, as reported in several studies [27, 32], can be due to the fact that diabetes mellitus (DM) and PDAC are associated diseases that have a complex and not completely understood relationship with each other [43]. On the one hand, long-standing DM is a low to moderate risk factor for PDAC [44]. Conversely, new onset of DM, especially over the age of 50, can be of paraneoplastic character and the first symptom of PDAC preceding other symptoms [45].

Increased levels of circulating branched-chain amino acids (BCAAs) are an early sign of PDAC onset, precede clinically evident cachexia, and are also elevated in

Table 1 PDAC biomarker publications that employed MS- or NMR-based metabolome analysis

Analytical method	Matrix	Research objective	Upregulated metabolites in PDAC (among others)	Downregulated metabolites in PDAC (among others)	References
LC-MS	Plasma	Identification of early diagnostic biomarkers	Isoleucine, leucine, valine	N/A	[24]
GC-TOF/MS, LC/IT/MS, LC-LTQ-MS	Plasma	Identification of early diagnostic biomarkers	Arachidonate, erythritol, cholesterol, N-methylalanine, lysine, deoxycholylglycine, cholylglycine, lysophosphatidylcholine (16:0), tauroursodeoxycholate, taurocholate, lysophosphatidylcholine (18:2), phosphatidylethanolamine (26:0), phosphatidylcholine (34:2)	Glutamine, hydrocinnamate, phenylalanine, tryptamine, inosine	[25]
LC-TOF/MS, GC-TOF/MS	Plasma	Identification of early diagnostic biomarkers	Methylguanidine, glutamate	Choline, betaine, 1,5-anhydro-D-glucitol	[26]
^{1}H-NMR spectroscopy	Urine	Identification of early diagnostic biomarkers	Acetone, hypoxanthine, o-acetylcarnitine, dimethylamine, choline, 1-methylnicotinamide, threonine, fucose, cis-aconitate, 4-pyroxidate, glucose, trimethylamine-N-oxide, aminobutyrate, tryptophan, xylose, trans-aconitate, 4-hydroxyphenylacetate, 2-hydroxyisobutyrate, taurine	Trigonelline, methanol	[27]

GC-QMS	Serum	Identification of early diagnostic biomarkers	Arabinose, ribulose	Valine, 2-aminoethanol, n-caprylate, threonine, nonanoate, methionine, creatinine, asparagine, glutamine, O-phosphoethanolamine, glycyl-glycine,1,5-anhydro-D-glucitol, lysine, histidine, tyrosine, urate	[28]
LC-MS, GC-MS	Serum	Identification of early diagnostic biomarkers	Mannose	Lysophosphatidylcholine (18:0 (sn1)), lysophosphatidylcholine (18:0 (sn2)), lysophosphatidylcholine (20:3 (sn2)), phosphatidylcholine (14:0/22:6), 1,5-anhydro-D-glucitol	[29]
GC-MS	Serum	Identification of early diagnostic biomarkers	Lactate, thiodiglycolate, 7-hydroxyoctanoate, asparagine, aconitate, homogentisate, N-acetyl-tyrosine, stearate, L-glycine, 3-hydroxybutyrate, L-glutamate, 4-hydroxyphenylacetate, palmitoleate, palmitate	Urea, octanoate, glycerate, decanoate, urate, 4-hydroxyproline, tartaric acid	[30]
FIA-MS/MS	Serum	Identification of early diagnostic biomarkers	Lanosterol, lignoceric acid, cholesterol 5α,6α epoxide, 1,2-dioleoyl-sn-glycero-3-phospho-rac-glycerol, erucic acid, oleanolic acid, taurochenodeoxycholic acid	Palmitate, 1-monooleoyl-rac-glycerol, oleoyl-L-carnitine	[31]
^{1}H-NMR spectroscopy	Urine	Identification of early diagnostic biomarkers	Acetoacetate, acetylated compounds, glucose, leucine, 2-phenylacetamide	Citrate, creatinine, glycine, hippurate, 3-hydroxyisovalerate, trigonelline	[32]

(*continued*)

Table 1 (continued)

Analytical method	Matrix	Research objective	Upregulated metabolites in PDAC (among others)	Downregulated metabolites in PDAC (among others)	References
LC-MS/MS, GC-MS, SPE-LC-MS/MS	Plasma	Differential diagnosis of PDAC (PDAC, chronic pancreatitis, non-pancreatic control patients (preoperative patients admitted for thyroid resection or hernia repair))	A biomarker panel of nine metabolites and CA19-9 was identified and validated: sphingomyelin (d17:1,C18:0), sphingomyelin (d18:2,C17:0), phosphatidylcholine (C18:0,C22:6), and isocitrate were increased in PDAC[a]; proline, histidine, sphinganine-1-phosphate (d18:0), pyruvate (additional: phosphoenolpyruvate), and ceramide (d18:1,C24:0) were decreased in PDAC[a]		[33]
FIA-MS/MS	Serum	Differential diagnosis of PDAC (PDAC, pancreatitis, healthy controls)	Amino acid-based metabolites in combination with CA19-9		[34]
CE-TOF/MS	Saliva	Differential diagnosis of PDAC (pancreatic cancer, breast cancer, oral cancer, periodontal diseases, healthy controls)	N/A	N/A	[35]
UHPLC-MS/MS, GC-MS	Tissue	Discovery of cancer progression markers	N/A	Linolenate (18:3n3 or 6), palmitate (16:0), margarate (17:0), stearate (18:0), linoleate (18:2n6), oleate (18:1n9), eicosenoate (20:1n9 or 11), 10-nonadecanoate (19:1n9)	[36]
FIA-MS/MS	Serum	Discovery of cancer progression markers	Tripentadecanoate, sphingomyelin (C24:1), symmetric dimethylarginine	Valine, lysine	[37]

[a]When determined as single biomarkers

Table 2 Metabolite profiling-based patent applications for metabolic biomarkers of PDAC

Analytical method	Matrix	Patent objective	Altered metabolites	References
^{1}H-NMR spectroscopy	Serum	Identification of early diagnostic biomarkers	Alanine, citrate, creatinine, formate, glucose, glutamine, histidine, lactate, and valine	[38]
Q-TOF, HPLC-MS/MS	Serum	Identification of early diagnostic biomarkers	Certain specific lysophosphatidylcholines, sphingomyelins, phosphatidylcholines, plasmenylphosphocholines, and plasmenylcholines	[39]
LC-MS/MS, GC-MS, SPE-LC-MS/MS	Plasma	Differential diagnosis of PDAC (PDAC, chronic pancreatitis, alcohol-induced liver cirrhosis)	Certain specific lipids, fatty acids, amino acids, and various hormones	[40]
LC-MS	Pancreatic cyst fluid	Differential diagnosis of pancreatic cysts[a]	Glucose and kynurenine	[41]

PDAC pancreatic ductal adenocarcinoma, *GC-MS* gas chromatography-mass spectrometry, *GC-QMS* gas chromatography-quadrupole mass spectrometry, *GC-TOF/MS* gas chromatography time-of-flight mass spectrometer, *LC-MS* liquid chromatography-mass spectrometry, *LC/IT/MS* liquid chromatography ion trap mass spectrometry, *LC-LTQ-MS* liquid chromatography linear trap quadrupole mass spectrometry, *LC-TOF/MS* liquid chromatography time-of-flight mass spectrometer, *CE-TOF/MS* capillary electrophoresis time-of-flight mass spectrometry, *FIA-MS/MS* flow injection analysis-tandem mass spectrometry, *UHPLC-MS* ultra-high-performance liquid chromatography-mass spectrometry, *^{1}H NMR* proton nuclear magnetic resonance spectroscopy, *SPE-LC-MS/MS* solid phase extraction-liquid chromatography-tandem mass spectrometry, *Q-TOF* quadrupole time of flight, *HPLC-MS/MS* high-performance liquid chromatography-tandem mass spectrometry

[a]Diagnosis and management of pancreatic cysts is clinically important because approximately half may have the potential for malignant transformation to pancreatic adenocarcinoma [42]

individuals with obesity, impaired fasting glucose, and type 2 diabetes which are common PDAC risk factors and/or comorbidities. It has been demonstrated that increased protein breakdown and a subsequent increase in plasma levels of BCAAs are early events in PDAC progression [24], suggesting muscle protein loss and/or paraneoplastic diabetes. These BCAAs and the breakdown products of muscle and adipose tissue may also serve as fuel sources for tumor growth [13].

Reprogramming of lipid metabolism represents another important metabolic feature of PDAC, as reported in several studies [25, 36], but this is also evident in individuals with the some of the most common risk factors and/or comorbidities of PDAC such as obesity. PDAC cells can use alternative lipogenesis routes to obtain fatty acids, whether through the uptake of extracellular lipids derived from diet, liver synthesis, or release from adipose tissue [46]. Additionally, the elevated requirement of cholesterol by PDAC cells can be supplied by *de novo* synthesis, receptor-mediated uptake of cholesterol (low-density lipoprotein receptor (LDLR)), or by

hydrolysis of cholesteryl ethers [47]. This emphasizes the role of high dietary intake and obesity as a risk factor of PDAC.

Further metabolomics studies in PDAC in combination with the integration of genetic information, such as that performed by Zhang et al. [36], are likely to improve disease management and may provide new insights and pave the way to new therapeutic strategies, urgently needed for this disease.

Potential Clinical Applications of Metabolomics in PDAC Management

Clinical metabolomics is expected to be a promising technology for precision medicine (also known as stratified or personalized medicine); however, cutting-edge metabolomics platforms are mainly found in specialized laboratories. Measurement of metabolites is well accepted in clinical routine use, and modern diagnostics rely heavily on the evaluation of pathologically altered metabolites, and the installed base for respective diagnostic platforms is high (i.e., urine test strips, clinical chemistry analyzer). The studies reviewed in this book chapter have identified potentially useful biomarker candidates that may be used in the future for the diagnosis of early PDAC either by improving the sensitivity and specificity of current tests or by substituting them.

Early Diagnosis

Developing a metabolomics-based biomarker for diagnosing a rare disease like PDAC is of special challenge. The benefit is clearly in early diagnosis when the disease is still in its resectable stage allowing for curative treatment. However, sample collections of early-stage patients are even more time-consuming. Access to large-sized and well-balanced case-control studies and prospective cohorts needs more time and resources compared to high-prevalence diseases. Statistical challenges need to be met for an excellent diagnostic performance in order to achieve sufficient positive and negative predictive values and develop a multivariate classification algorithm for a multi-panel biomarker. For example, Kobayashi et al. [28] constructed a GC-MS serum-based diagnostic model and then validated it via the stepwise variable selection method and subsequent multiple logistic regression analysis. The sensitivity of the new model was 77.8% compared to CA19-9 = 55.6% and CEA = 44.4%, in resectable PDAC (stages 0 to IIB). Another recent study [48] showed that a lipid called phosphatidylcholine-594 distinguishes PDAC from control with a sensitivity of 85% at a fixed specificity of 90% and is on the market as a PDAC risk assessment test (PanaSee™, Phenomenome Discoveries Inc.). Since these diagnostic models had a higher sensitivity than CA19-9, applying them in a clinical setting could reduce the incidence of missed malignant changes, reduce unnecessary and expensive follow-up diagnosis or surgery due to a false-positive diagnosis, and lower the psychological burden of being falsely diagnosed with a deadly disease.

Differential Diagnosis

Patients with chronic pancreatitis have a much higher risk for the development of PDAC than the general population. Established diagnostic methods such as CA19-9 (tumor surface marker Sialyl-Lewis A) suffer from insufficient clinical performance, and both diseases present with similar symptoms. Therefore, the differentiation between both diseases remains a clinical challenge. In a multicenter discovery case-control study, subjects were prospectively recruited with either PDAC, chronic pancreatitis, or non-pancreatic control patients (preoperative patients admitted for thyroid resection or hernia repair), and plasma samples were investigated by metabolomics [33, 49]. A biomarker signature of nine metabolites and CA19-9 was identified and validated in independent cohorts for the differential diagnosis between PDAC and chronic pancreatitis [33]. From these results, a targeted quantitative assay (MxP® PancreasScore) was developed that simultaneously quantifies polar and lipid metabolites after extraction and dansylation of samples by LC-MS/MS analysis (personal communication). Applying a fixed diagnostic cutoff value of $\geq$0.608 for the pancreatic biomarker score, an 81% sensitivity for PDAC was achieved in combination with a specificity of 94% for chronic pancreatitis and 91% for non-pancreatic controls, respectively (Fig. 3). Routinely utilizing this biomarker assay and the underlying biomarker signature can, inter alia, help to more accurately distinguish PDAC from chronic pancreatitis and thus support physicians in choosing optimized therapeutic options.

Conclusion

Metabolomics, a high-throughput global metabolite analysis, is a developing field, and substantial evidence has demonstrated its emerging role in PDAC management. Advances in metabolomics along with the novel strategies to analyze, understand, and construct the metabolic pathways open a window of opportunity in a very effective manner. The systems biology approach in biomarker investigation may allow for a deeper understanding of the metabolic path mechanisms of PDAC [50]. Such an approach does not focus on identifying a single target or mechanism of an observed phenotype, but rather seeks to identify the biological networks or pathways that connect the differing elements of a system [51]. Thus, the systems biology approach in combination with metabolomics may lead to the discovery of panels of metabolites that more accurately capture the disease status and help acquire information valuable for individualized clinical care [52].

So far, the different applications of metabolomics have resulted in promising findings but are not sufficient to change current clinical practice. More studies are needed in the future. Clinical trials are ongoing, testing different combinations of drugs that target specific metabolic pathways associated with PDAC. Metformin combined with PDAC chemotherapy (i.e., gemcitabine) is currently being tested in several clinical trials on metastatic PDAC patients. The direct targets of metformin

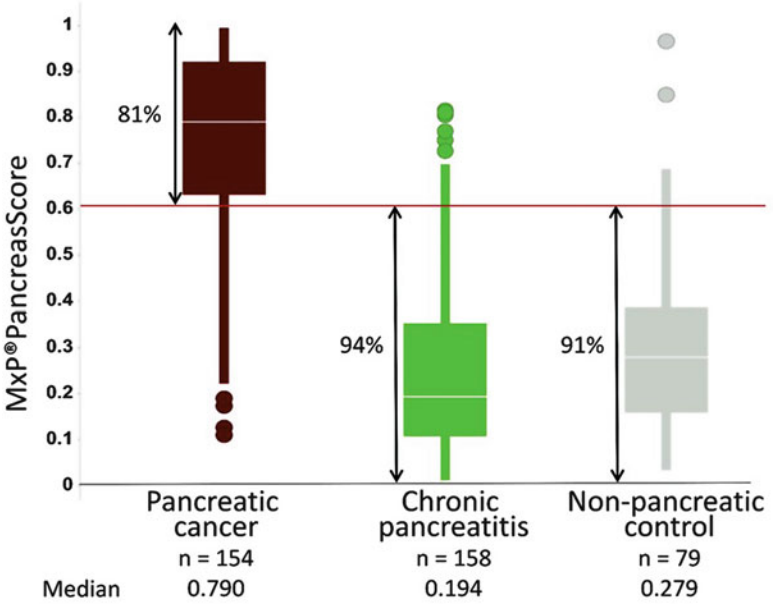

Fig. 3 MxP® PancreasScore generated with metabolites and CA19-9. Box plots give median, upper quartile and lower quartile by the box, and the upper adjacent and lower adjacent values by the whiskers. The upper adjacent value is the largest observation that is less than or equal to the upper inner fence, which is the third quartile plus 1.5-fold interquartile range. The lower adjacent value gives the corresponding value for downregulation. The diagnostic cutoff of the pancreatic biomarker score was set to $\geq$0.608. This translates in a sensitivity for PDAC of 81% and a specificity for CP of 94%, respectively, non-pancreatic controls of 91%.

are not well understood. It does, however, target key metabolic pathways of PDAC cells, giving it potential therapeutic value [13].

Future research frontiers in cancer metabolomics offer great promise. For example, the surgical iKnife (intelligent knife) could help surgeons distinguish between tumor and healthy tissue in the operating room. The iKnife couples existing electrosurgical equipment with a technique known as rapid evaporative ionization mass spectrometry to provide analyses in near real-time by *in vivo* analysis of the aerosol ("smoke") released during electrosurgical dissection [53]. Since tumors have different chemical signatures than healthy tissue, analysis of these signatures via mass spectrometry could help cancer surgeons remove tumors but leave suitable margins of healthy tissue intact, providing a faster, more data-rich alternative to sending samples to a pathologist during surgery.

Stable isotope-labeled metabolites could represent a suitable approach to increase the current basic understanding of the metabolic dependencies of PDAC cells. Isotope labeling is often used to trace pathways within metabolic networks [54, 55]. Another beneficial experimental method for cell culture metabolomics analysis involves stable isotope labeling followed by either MS or NMR measurement. This approach enables pathway tracing, easier metabolite assignment, and

metabolic flux measurements. Isotopic labeling has previously enabled detailed determination of pathways leading to the production of specific metabolites and the development of highly accurate mathematical models of these pathways [56].

MS-based metabolite imaging uses radioactively labeled metabolites or their precursors for *in vivo* imaging that can be used to confirm, in intact living systems, preclinical and *in vitro* assessments. A limitation of LC-MS, CE-MS, or GC-MS methods is the loss of spatial information that results upon metabolite extraction from homogenized samples. Metabolomics imaging technologies can be, therefore, an important alternative and provide information on the spatial distribution of metabolites within tissues. MALDI imaging is the most widely used MS-based tissue imaging approach [57]. MALDI matrix is typically applied to the sample (i.e., tissue) either by spotting or spraying, and images are generated by raster scanning the laser over the sample. Composite images are constructed by mapping the distribution and abundance of ions within the sample.

Although, direct translation of metabolite findings toward management of PDAC medicine is still in its infancy, the advance of analytical metabolite profiling technologies will enable new diagnostic assays with improved sensitivity and specificity over the current conventional biomarkers to be implemented in routine laboratories.

Cross-References

- ▶ Development of Novel Diagnostic Pancreatic Tumor Biomarkers
- ▶ Development of Novel Therapeutic Response Biomarkers
- ▶ Diagnostic Biomarkers
- ▶ Metabolism in Pancreatic Cancer

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Circulating Tumor Cells

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Abstract

Analysis of cellular and molecular components of tumor origin detectable in the bloodstream, so-called liquid biopsies, is demonstrating potential to support management of cancer patients. Development of sensitive technologies enables detection, isolation, and downstream analysis of both circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) from the blood of patients with various malignancies, in a minimally invasive way, allowing temporal and spatial monitoring of the clinical course of the disease. This is particularly significant in cancers such as pancreas cancer, a particularly aggressive disease with limited treatment options and poor outcomes, where serial biopsy is challenging. CTC enumeration; genomic, transcriptomic, and proteomic analysis; as well as in-depth sequencing of ctDNA may define a comprehensive molecular and genetic landscape of pancreatic cancer and provide a set of novel biomarkers for screening, diagnosis, prognosis, and response assessment. A number of pilot studies have been conducted to assess the role of liquid biopsies in the setting of pancreatic cancer. Although results so far seem promising, more extensive studies are required to establish the clinical utility of CTCs and ctDNA in developing a personalized approach for the management of this malignancy.

Keywords

Circulating tumor cells · Circulating tumor DNA · Liquid biopsy · Pancreatic cancer · Biomarkers

Introduction

A major challenge in managing patients with pancreatic cancer is the need for rapid assessment of disease stage to design an optimal treatment plan. Liquid biopsies, including circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) to

diagnose cancers, stratify cancer patients for personalized therapies, and monitor tumor evolution and response to treatment are increasingly being studied and are beginning to be clinically implemented. Notably, the first ctDNA test for lung cancer treatment with an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor was approved by the Food and Drugs Administration (FDA) and European Medicines Agency (EMA) in June 2016 and January 2015, respectively. The potential for liquid biopsies to assist the management of pancreatic cancer, a disease where tumor biopsy is particularly challenging, is reviewed throughout the following sections.

Management of pancreatic cancer, and in particular pancreatic ductal adenocarcinoma (PDA), presents an urgent medical need. PDA is the 10th most common solid cancer in the United States and ninth in Europe, and the second most common gastrointestinal malignancy. However, due to late diagnosis, aggressive disease progression, and limited treatment options, PDA is currently the fifth leading cause of cancer-related death in Europe and the fourth in the United States [1], [2].

Premalignant stages of PDA are classified as pancreatic intraepithelial neoplasia (PanIN). Common genetic aberrations such as activating mutations in the oncogene KRAS and loss of function in the tumor suppressor genes CDKN2A, SMAD4, and TP53 have been identified early during PDA development. Genomic analysis in fully developed PDA and metastatic lesions has further identified ~200 less frequently occurring genetic aberrations. When grouped according to the pathways affected by these mutations, new therapeutic opportunities may emerge [3], [4].

Most patients with PDA are diagnosed at advanced stages and only one in five patients is eligible for surgery with or without adjuvant chemotherapy, which is the only curative treatment strategy available at the moment. Unfortunately, 66% of these patients will experience local recurrence, distant metastasis, or both, leading to a median overall survival in the range of 28 months [5]. The remaining 80% of patients present at the metastatic stage with a dismal 5 year overall survival (OS) of only 2% [2] and are offered standard of care chemotherapy (either gemcitabine monotherapy or combination chemotherapy regimens: gemcitabine and *nab*-paclitaxel or FOLFIRINOX [5-fluorouracil, oxaliplatin, and irinotecan]) with modest impact on overall survival. Although new treatment modalities have been introduced recently, these still only improve OS by weeks to months.

The Metastatic Cascade and Timing of Metastatic Events in Pancreatic Cancer

Development of metastatic disease accounts for 90% of cancer-related deaths in solid tumors and is a common phenomenon during the natural course of PDA. The most common site of metastasis is the liver followed by the peritoneum, lung, and abdominal lymph nodes. Less commonly PDA metastasizes to the adrenal glands, bones, and thoracic lymph nodes. Metastasis is driven by accumulation of genetic and/or epigenetic aberrations, which provide cells with enhanced capabilities for migration, tissue invasion, survival in new microenvironments, and the ability to

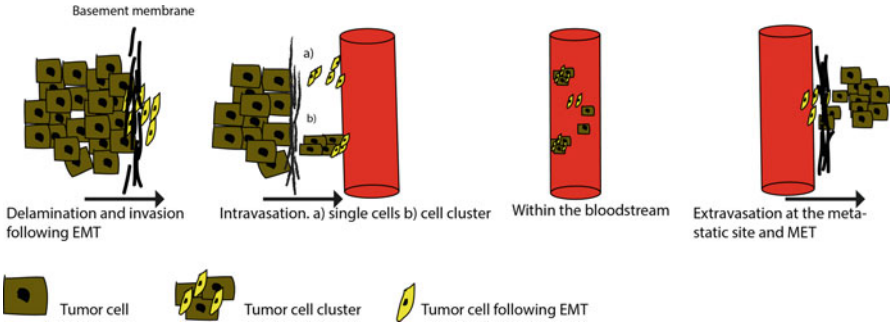

Fig. 1 The metastatic cascade. *Arrows* indicate direction of move. EMT: epithelial to mesenchymal transition. MET: mesenchymal to epithelial transition

seed distant organs and form secondary tumors [6]. Key events underpinning tumor cell metastasis include the delamination and intravasation of tumor cells into the vascular system. Provided they survive in the circulation, tumor cells then extravasate and invade distant organs to form secondary tumors. Together this is also known as "the metastatic cascade" (Fig. 1).

Circulating tumor cells (CTG), the likely harbingers of metastasis, are identifiable in the circulation of genetically engineered murine models (GEMMs) of PDA, even at early stages of disease development (PanIN). This suggests that the metastatic cascade may be initiated early and that the primary and secondary tumors evolve separately in different microenvironments [7]. Clinically this is translated into the synchronous presence of primary and metastatic tumors at diagnosis. Genetic analysis of matched primary and metastatic human tumors provided insight into intratumoral heterogeneity and the acquisition of mutations during the metastatic cascade.

The estimated timeline for PDA metastasis [6] combined with the poor median OS of 3–6 months from time of diagnosis suggests that most patients are only diagnosed after the dissemination of the first metastatic cell. Consequently, newly diagnosed patients likely already harbor occult micro-metastases undetectable by current imaging methodologies. Furthermore, the genetic and epigenetic changes accumulated by cells during and after this period cause remarkable heterogeneity. Firstly, the population of cells that form metastatic deposits is significantly altered compared to the original tumor-initiating cell (TIC) due to clonal evolution in the primary site giving rise to distinct subclones [6]. Further epigenetic changes are accumulated after the formation of metastatic deposits [8], even though metastases share common driver mutations with subclones within the primary tumor [9]. The heterogeneity of metastasis-initiating cells is also evident by their ability to seed various organs with different capillary bed structures and adjacent microenvironment. Moreover, there is ongoing clonal evolution after overt metastases formation and evidence that secondary metastases also harbor TICs, which in turn might enter the circulation to seed further sites [10].

This overall heterogeneous tumor burden has direct implications for the management of metastatic pancreatic cancer. Firstly, certain cell subpopulations such as TICs and those cells undergoing EMT are thought to be relatively chemoresistant [11], [12]. Furthermore, due to inter- and intra-patient tumor heterogeneity, patients respond differently to standard treatments and also after initial response, rapid progression occurs. In addition, the aforementioned diversity makes selection of patients for clinical trials highly inefficient and is likely to result in erroneous assessment of the efficacy of potentially useful agents.

There is a compelling argument that tumor heterogeneity within a patient with metastatic disease might be best reflected in the bloodstream where the cells that have completed the first steps in the metastatic cascade (migration, tissue invasion, and intravasation) can be sampled and assessed. Although it is not clear at the time of sampling which of the sampled CTCs will successfully complete the latter stages of the metastatic cascade and initiate secondary tumors, studying CTCs and the biology of metastatic dissemination will lead to better understanding of the complex biology that underpins pancreatic cancer metastasis. CTCs also have potential as prognostic tools and a source of biomarkers to support the development and selection of therapy.

Circulating Tumor Cells

The history of CTC research began in 1869 by Thomas Ashworth, an Australian physician who observed "...cells identical with those of the cancer itself..." in the blood of a patient with metastatic breast cancer at autopsy [13]. He compared the morphology of the cells in the blood to those from different lesions and concluded that "One thing is certain, that if they [CTCs] came from an existing cancer structure, they must have passed through the greater part of the circulatory system to have arrived at the internal saphena vein of the sound leg."

Eighty-six years later, the interest in CTC detection was revived when Engell published a report describing "the occurrence of cancer cells in the peripheral blood and in venous blood draining the tumor area at operation" [14]. During the following decade, 40 groups described CTC detection using cytological methods; reports that were notable for high CTC numbers. However, there were false-positive counts, as CTCs were often confused with hematopoietic cells, particularly megakaryocytes. Improvements in CTC detection came in 1980s with the emergence of immunocytochemistry. Despite advances in methodology for the detection and characterization of CTCs, their clinical utility was not demonstrated until 2004, when Cristofanilli and colleagues showed that in metastatic breast cancer, increased number of CTCs at baseline and after initiation of treatment is associated with worse progression free and overall survival [15].

The first attempt to detect CTCs in pancreatic cancer was reported in 1996, when Funaki et al. used reverse transcription-polymerase chain reaction (RT-PCR) to detect carcinoembryonic antigen (CEA) mRNA as an indicator of the presence of

adenocarcinoma cells in the blood of patients diagnosed with pancreatic cancer [16]. To date, multiple studies using a variety of methods have been reported trying to establish the optimal method to isolate and characterize pancreatic cancer CTCs and to determine their clinical significance.

The natural history of CTCs can be partitioned into four steps that overlap with key events of the metastatic cascade. Each of these steps is associated with specific changes at the molecular and phenotypic level.

Step 1: Delamination and Intravasation

The first step involves detachment of tumor cells from the primary site and entry into the blood circulation. Acquisition of a migratory phenotype, possibly through epithelial to mesenchymal transition (EMT), allows individual tumor cells to separate from the bulk of the primary tumor. Key to this process is the loss of cell-cell adhesions, for example, through downregulation of E-cadherin expression, a protein critically involved in the establishment of adherens junctions between epithelial cells. In parallel, expression of mesenchymal markers, such as α-SMA, FSP1, vimentin, and desmin, are typically increased. Moreover, during EMT epithelial cells change their shape, lose their apical-basal orientation, and acquire the more mobile mesenchymal phenotype [17]. These changes are accompanied by production of metalloproteinases, which disintegrate the basement membrane and the extracellular matrix, together with tumor neovascularization that facilitates tumor cell invasion into the circulation [17].

An alternative, but nonexclusive, theory supports the "collective migration" of tumor cells, where malignant cells that maintain their epithelial phenotype and cell-cell contacts migrate in cohorts "led" by mesenchymal cells. In co-cultures of squamous cell carcinoma (SCC) cells and fibroblasts, fibroblasts in contact with and at the leading edge of SCC cell clusters paved the way by remodeling the matrix through both physically generated forces and MMP-mediated degradation. The epithelial cells then follow, keeping together via regulated cytoskeletal forces between them [18]. Consistent with this mechanism, the role of EMT in pancreatic cancer cell invasion has been questioned after data showing that deletion of the EMT-inducing transcription factors Twist or Snail in mouse models of PDA failed to abrogate the migratory and invasive potential of the cancer cells [19]. In contrast to the above active form of migration, cell clusters may also passively separate from the primary and enter into the bloodstream [20].

Step 2: Within the Bloodstream

Once in the circulation, cancer cells travel to distant organs along with billions of normal blood cells. The half-life of CTCs in the blood is estimated in the range of 1–2.4 h [21]. CTC survival in the bloodstream is limited by apoptosis, induced by deprivation of stroma-derived growth and survival signals, shear stress in the

circulation, capture and apoptosis within the lungs [20], and clearance by liver Kuppfer cells [22]. CTCs may undergo immune attack by both the innate and possibly the adaptive immune system, orchestrated by natural killer cells (NK cells) and T-lymphocytes, respectively [23].

The assumption is that a fraction of CTCs must survive in the circulation in patients with metastatic cancer, but whether all surviving CTCs are able to form secondary tumors is highly questionable. In an experimental mouse model, where xenografts were developed by injection of CTCs isolated from patients with metastatic breast cancer, immunohistochemistry shows that TICs are likely $CD44^+$ $CD47^+MET^+$ [24] indicating that subsets of cells with higher tumor-initiating capability exist within this population. Such TICs with migratory capabilities have been identified in PDA as $CD133^+/CXCR4^+$ cells [12].

Step 3: Seeding Distant Organs and Metastatic Tumor Formation

Upon arrival at distant organs CTCs extravasate through the capillary walls. As demonstrated in mouse models, cells with a permanent mesenchymal phenotype do not cause development of metastatic tumors. Instead cells undergo the reverse process of mesenchymal to epithelial transition (MET), which transforms mesenchymal circulating cells to epithelial disseminated cells. This is achieved by down-regulation of transcription factors that promote EMT, and potentially explains why metastatic deposits from epithelial primaries demonstrate epithelial histology [25].

CTCs face a new microenvironment at the secondary site. Direct visualization by in vivo videomicroscopy of intraportally injected melanoma cells in an experimental mouse model revealed that only a small fraction of extravasated cells will actively proliferate to form micro-metastases and even fewer micro-metastases result in overt tumor colonies. Another subset of cells remain in the host microenvironment as single cells in dormancy showing neither proliferation nor apoptosis as assessed by Ki67 staining and TUNEL assay, respectively [26]. Apart from these quiescent cells, dormancy can also be observed in dividing cells that are unable to expand as the rate of proliferation is counteracted by equal rate of cell death. Mechanisms responsible for this process include inefficient angiogenesis and control by the immune system [27].

Critical to cancer cell survival in the metastatic host organ is the generation of promoting signals by a “metastatic niche,” a term referring to the outcome of the interaction of stromal cells, extracellular matrix components, and cell signals that enhance survival and self-renewal of extravasated cancer cells [25]. In a mouse model of pancreatic cancer liver metastasis, it has been proposed that this niche is induced by exosomes derived from the primary tumor. Exosomes are membrane vesicles of endocytic origin containing proteins, DNA, mRNA, and microRNA that are taken up by the liver and induce changes in the microenvironment, such as fibronectin production and recruitment and deposition of bone marrow-derived macrophages and granulocytes, ultimately resulting in enhanced liver metastatic seeding. Importantly, the above described changes and initiation of the metastatic

niche are thought to commence before primary tumorigenesis, at the stage of preneoplastic pancreatic lesions [28].

Having secured their survival and also self-renewal capacity via interaction with the metastatic niche, disseminated tumor cells (DTCs) with tumor-initiating capacity activate adaptive programs that provide them with phenotypic characteristics enabling host organ colonization. For example, potential metabolic adaptations may be required for DTCs to overcome the increased oxidative stress at visceral organs [29]. Once DTCs acquire the new traits, they can exploit the interaction with host stromal cells, to destroy the host organ's extracellular matrix, leading to creation of space for the cancer cells to grow.

Step 4: Self-Seeding

CTCs may not originate solely from the primary tumor. In breast cancer models, tumor cells escape metastatic sites to reenter the circulation and reinfiltrate the primary tumor site responding to chemoattractants, a process known as "tumor self-seeding," where in contrast to distant sites, there is little additional adaptation required for further expansion. This process recruits aggressive CTC populations, such as CTC that were shed from metastatic sites after accumulation of additional genetic aberrations, with the result that the primary is now reseeded with more aggressive and heterogeneous tumor cells. These recently recruited cells can interact with the tumor stroma leading to release of growth signals that promote angiogenesis and invasion with an ultimate outcome of local-regional progression and enhanced heterogeneity [30].

Methods for CTC Detection

Overview

CTCs are rare cells with an average of one CTC per 10^6–10^8 blood cells in the circulation. This rarity makes the detection and isolation of CTCs technically challenging. CTC detection, enrichment, and isolation assays exploit various defining characteristics of tumor cells to discriminate and isolate them from the overwhelming number of blood cells in the sample. Most CTC workflows start with enrichment of CTCs followed by CTC detection and enumeration and then a second step to isolate and analyze single CTC molecular profiles. However, not all steps are incorporated within every platform, depending on the purpose of the study and the complexity of the method used. More recent approaches dispense with the enrichment step (where CTC losses can occur) and detect, enumerate, and characterize CTCs within the entire blood sample. This "no cells are lost" approach may be critical for minimal residual disease monitoring and for early detection. Also central to the utility of a CTC assay is the portability of the blood sample, that is, the time from blood draw to CTC enrichment and analysis before the sample has degraded.

This is especially important for multisite studies and the CellSave® preservative tube that allows 4 days from blood draw to analysis at room temperature was a significant step forward in the field.

The worth of a CTC platform is commonly measured by means of CTC recovery rate, enrichment, purity, and throughput. Recovery rate is the fraction of the tumor cells present in the sample often derived using a "spike-in" of a known number of cultured tumor cells. Purity refers to the ratio of bona fide CTCs to other cell types recovered by the platform, and enrichment is the factor by which output purity has increased compared to the input purity. Finally, throughput refers to the volume of blood or number of cells that are processed by the platform within a given time. A high performance platform therefore is considered one which combines high enrichment, purity, recovery rate, and throughput [31]. Another attractive feature is the ability of the platform to enrich and or isolate viable, intact CTCs suitable for culture in vitro or in vivo.

The technologies that have been employed in pancreatic cancer CTC studies are discussed followed by the clinical information they generated.

CTC Enrichment

Methods for enriching CTCs can be broadly divided into marker-dependent and marker-independent approaches. Marker-dependent CTC enrichment is based on expression of cell surface molecules that distinguish CTCs from white blood cells, while marker-independent methods exploit the different physical properties of CTCs compared to blood cells, such as size, inertia, dielectric charge, and density.

Marker-Dependent CTC Platforms

Typically multiparameter immunofluorescence is used for positive and negative cell selection using epithelial cell adhesion molecule (EpCAM), a transmembrane glycoprotein widely and exclusively expressed by epithelial cells and carcinomas [17], and/or alternative tumor associated surface markers, and CD45, a glycoprotein expressed by nucleated hematopoietic cells, to exclude white blood cells (WBC), respectively [17]. Vital to the appropriate assignment of mesenchymal CTCs that may have downregulated epithelial markers via EMT is the inclusion of a marker to exclude circulating endothelial cells (that usually outnumber CTCs in a blood sample). Specifically, increasing the staining assay complexity to assess CD31 or CD105 and vimentin expression allows identification of CD31/CD105-negative, vimentin-positive, epithelial marker-negative cells to be assigned as likely mesenchymal tumor cells. Even then isolation and genomic evaluation of putative CTCs is warranted to confirm tumor origin.

The CellSearch CTC platform (recently acquired by Menarini-Silicon Biosystems) led the CTC research field and is considered the "gold standard" for other platforms to benchmark against in terms of robustness. It has been extensively used in the setting of various malignancies and is FDA-approved for the prognosis and monitoring of metastatic breast, colorectal, and prostate cancers. Patient blood

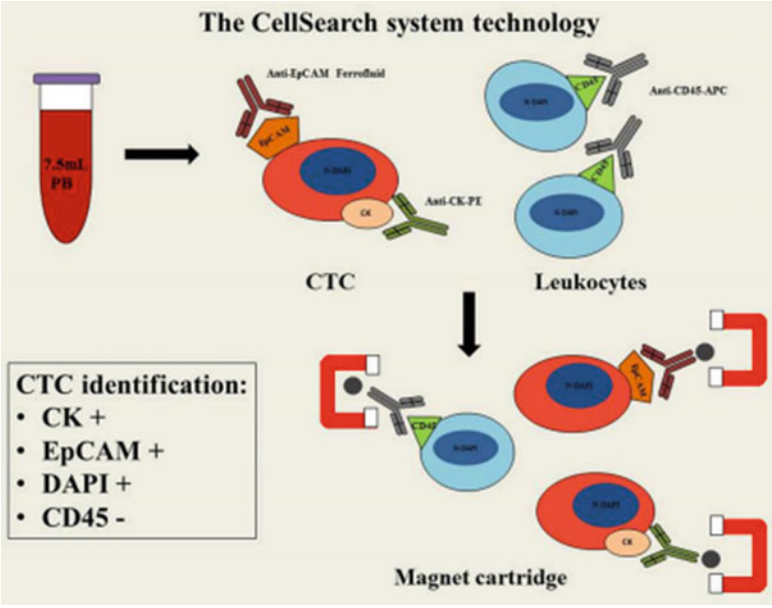

Fig. 2 The CellSearch workflow. *PB* peripheral blood. (Figure as originally published in Ref. [122])

(7.5 ml) is mixed with ferrofluid particles covered with anti-EpCAM antibodies that bind to epithelial CTCs. These ferrofluid particle-CTC complexes are then separated from other cellular components by application of a magnetic field. The sample containing the enriched CTCs is then stained with immunofluorescent monoclonal antibodies against pan-cytokeratin (CK-8, CK-18, and CK-19), CD45 to exclude white blood cells, and a nuclear stain, 4,6-diaminidino-2-phenulindole (DAPI) in order to identify nucleated cells. Samples are then imaged and CTCs are detected as CK+/CD45−/DAPI+ cells (Fig. 2).

In patients with metastatic pancreatic cancer, CTCs were detected less frequently and in smaller numbers compared to other malignancies [31]. CellSearch was also used in the locally advanced pancreatic cancer, to assess the prognostic role of CTCs [32]. However, despite its widespread use, CellSearch has a significant disadvantage, as CTCs that have undergone EMT may have lost epithelial surface markers and remain undetected. As outlined above, these mesenchymal CTCs are likely to hold greater potential to initiate metastasis.

In an attempt to maximize yield and capture pancreatic CTCs that may not express EpCAM, another immunoaffinity approach used immunomagnetic Dynabeads coupled with both anti-EpCAM and anti-MUC-1 antibodies, as MUC-1 is a marker with high sensitivity and specificity for PDA [33]. This method is similar in concept to CellSearch, but following application of magnetic field, in addition to EpCAM expressing cells, it also captures EpCAM negative cells that express the surface marker MUC-1 [34].

Negative Enrichment Approaches

Negative selection via immunoaffinity-based depletion of nontarget cells from a blood sample results in the capture of intact, viable, unmodified CTCs for further downstream analysis. This approach could potentially enrich multiple CTC subpopulations, addressing CTC heterogeneity, but confirmation of tumor origin of the unlabeled cells would be mandatory.

A study using antibodies against multiple markers expressed in nontumor cells resulted in high CTC enrichment efficiency and a mean recovery rate of 82 ± 10%, in "spike-in" experiments which included pancreatic cancer cells. The antibody cocktail used targeted multiple categories of blood cells, specifically, anti-CD45 to target leukocytes, anti-CD16 to target natural killer cells and neutrophils, anti-CD19 to target B-cells, anti-CD163 to target monocytes and macrophages, and anti-CD235a to target red blood cells [35]. Other studies used anti-CD45-only coated magnetic beads to capture and remove WBCs in order to enrich for CTCs independently of EpCAM expression [36], [37].

Marker-Independent CTC Platforms

Size-Based Methods

The majority, but not all, CTCs are larger than WBCs, a difference that has been exploited by many platforms that capture CTCs on microfilters. This approach offers the advantage of isolating cells without modifying their morphology as long as the pressure placed upon them during filtration is optimized. However, contamination by trapping nontumor cells on the filters reduces purity. Two similar filtration methods have been applied to pancreatic cancer: isolation by size of epithelial tumor cells (ISET) [38] and ScreenCell [39], [40].

The ISET platform consists of a 10-well plastic reservoir above a polycarbonate membrane perforated with 8 μm cylindrical pores. After red blood cell lysis, blood is loaded on each of the wells and undergoes filtration by applying regulated suction. CTCs are fixed onto the membrane and are then stained, enumerated, and further analyzed. In a study directly comparing CellSearch with ISET, CTCs were detected in more pancreas cancer patients and in greater numbers per patient by ISET. There was no correlation between the two platforms regarding the number of detected CTCs, suggesting that the two different methods may capture separate subpopulations of CTCs [41]. In the ScreenCell device, blood flows through a microporous filtration membrane and CTCs are captured via low-pressure vacuum-filtration on small metal-rimmed filters.

Density Gradient–Based Methods

Here, blood samples are layered over a resolving medium (Nycoprep [42], Monopoly [43], Ficoll-Isopaque [44]), followed by centrifugation, which separates the CTC-containing peripheral blood mononuclear cell (PBMC) layer, based on density properties. The presence of CTCs in this cell layer can then be detected either by mRNA extraction and RT-PCR for cancer-specific genes or assigned via immunocytochemistry.

Dielectrophoresis Enrichment (DEP)

Due to their unique phenotype and cellular constituents cells of different origins have different electric properties, and this property can be utilized by application of an electric field, resulting in controlled movement of individual cells. The dielectrophoresis method exploits this property to separate and enrich CTCs from normal blood cells. In pancreatic cancer, a study has combined DEP with immunocapture in a Hele-Shaw flow cell to enhance the purity of the captured cells. Application of an electric field near the antibody-coated immunocapture surface in the device achieved isolation of cells from various pancreatic cell lines with high purity, by attracting tumor cells and repelling noncancerous blood cells in spike in experiments [45].

Microfluidic Methods

So-called Lab-on-a-chip devices are designed to encompass several laboratory functions on a microchip. Microfluidic "lab-on-a-chip" devices can process blood down to microliter amounts and by utilizing high throughput arrays are useful for rapid CTC enrichment from patient blood samples. The internal surface of such devices can be functionalized using coatings of antibodies against cell surface markers for positive selection of CTCs.

The CTC-CHIP consists of an array of micropillars conjugated with anti-EpCAM antibodies, where capture of CTCs on the surface of micropillars is achieved, as shown in a study where CTCs were isolated from >99% of patient samples with sensitivity of 99.1% and specificity of 100% and high reproducibility [46]. In an attempt to further increase efficiency a staggered herringbone design (geometrically enhanced mixing Chip, GEM-CHIP) was adopted, which by inducing micro-vortices leads to increased rate of cell-chip surface interaction and greater capture efficiency [47].

CTC-iCHIP

The CTC-iCHIP, the most advanced microfluidic device, can be utilized to enrich CTCs both from epithelial and nonepithelial malignancies, as it can operate either in a surface marker dependent or independent marker (positive and negative selection). It combines several functions exploiting different cell properties, incorporated in three sequential steps on the device. During the first step, whole blood containing either immunomagnetically labeled CTCs (positive selection) or WBCs (negative selection) flows through a set of micropillars along with buffer, where based on their size, red blood cells, platelets, and other blood components are separated and discarded. The remaining CTCs and WBCs enter the second step where they are aligned in single file using inertial focusing. Finally by application of a magnetic field, CTCs are separated and collected for further analysis. The CTC-iCHIP was assessed in spike-in experiments and in a study with blood from patients with

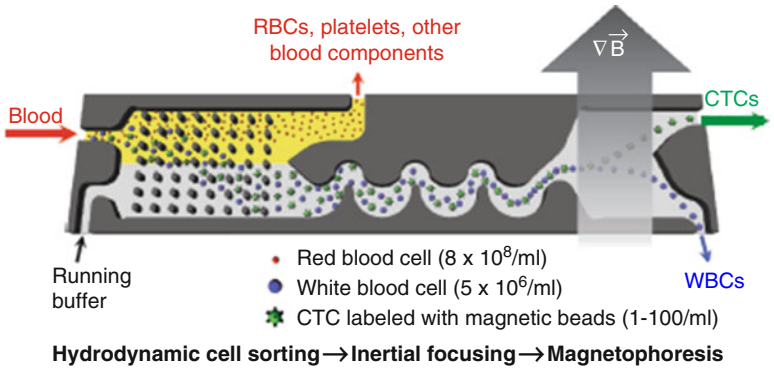

Fig. 3 The CTC-iCHIP workflow (positive selection mode). Following immunomagnetic bead labeling, whole blood flows through a set of micropillars along with buffer. In the first step nucleated cells are separated from other blood components and enter a nonsymmetric, serpentine-shaped microchannel where they are aligned in single file by inertial focusing. In the final step, labeled cells are deflected and collected following application of magnetic field [48]

prostate, pancreatic, breast, lung, and colorectal cancer and yielded CTCs in suspension with high efficiency and purity, suitable for further analysis (Fig. 3) [48].

Functional Assays

Functional assays for CTC enrichment exploit the ability of cancer cells to adhere to a tissue or tumor microenvironment mimic, referred to as cell adhesion matrix (CAM). The Vita-Assay enriches CTCs, including TICs, regardless of primary tumor origin, cell size, morphology, or surface markers, based only on their capacity to invade CAM. Therefore, theoretically it captures the most aggressive cells that hold the greatest metastatic potential. Captured cells are viable cells that can undergo further downstream analysis. Importantly, CAM-captured cells can also ingest CAM. Fluorescently labeled CAM allows for direct visualization of CAM+ cells. This method has been used to enrich CTCs form patient blood including patients with pancreatic cancer [49]. Combination of this platform with fluorescence activated cell sorting (FACS) resulted in increased capture purity in another study of patients with metastatic PDA among other metastatic cancers [50].

CTC Detection

CTC detection, the process by which enriched cells are assigned as CTCs, is achieved based on protein expression profiles or genomic analysis. Protein markers can either be generic, showing the epithelial or mesenchymal origin of a cell, or more specific to tumor tissue of origin suggesting the assigned cell is a CTC candidate. A combination of phenotyping and genotyping is preferable to confirm the identity as a CTC.

Immunocytochemistry

CellSearch utilizes fluorescently tagged antibodies to pan-cytokeratin (CK-8, CK-18, CK-19) as an epithelial marker and to CD45 as WBC marker in order to detect EpCAM-based enriched CTCs, as outlined above. AE1/AE3 is an additional pan-cytokeratin marker which has been used to detect epithelial cells in the blood and bone marrow from patients with pancreatic cancer [42]. CTCs with tumor-initiating capacity were detected by staining with antibodies CD133, CD44, and ALDH to detect "cancer stem cells" [38]. CTCs undergoing EMT were also detected, after staining with antibodies against the EMT marker zinc finger E-box binding homebox 1 (ZEB1) [40]. Immunofluorescent staining was also used to detect MUC1 [51] and MUC4 (another marker present only in pancreatic malignant and premalignant tissues and not in healthy pancreas) expressing pancreatic CTCs [52]. Finally the combination of carbohydrate antigen (CA) 19–9 and CK 8/18 was used to detect apoptotic pancreatic CTCs following fluoropyrimidine treatment, as coexpression of these markers on cells was found to correlate with morphological changes and apoptosis-indicating staining markers [53].

Genomic Confirmation of CTC Assignment

Genomic analysis can be used to confirm CTC identities based on genetic alterations that they share with the primary tumor and are absent in normal somatic cells. This method was used to detect CTCs in a Patient -denied explant (PDX) mouse model of PDA, where single cell genomic analysis of CTCs revealed that they had the same KRAS G12 V mutation as the primary tumor [54]. KRAS mutation is a particularly useful marker in detecting pancreatic CTCs, as it is present in >90% of pancreatic adenocarcinoma. As a consequence, isolated cells positive for this mutation can be reliably identified as pancreatic cancer cells.

An alternative approach is to detect the presence of pancreatic tumor-specific mRNA by RT-PCR after RNA extraction from cells that are present, for example, in the PBMC cell layer following centrifugation in the enrichment step. This approach, even though does not allow for direct cell visualization, provides indirect evidence of the existence of cancerous among nonmalignant cells, like blood cells. mRNA markers that have been used in studies of pancreatic cancer include carcinoembryonic antigen (CEA) [43], CK20 [44], EpCAM [55], and CK19 [56].

Single-marker approaches do not address tumor heterogeneity. A multimarker approach employing RT-PCR analysis of multiple genes, namely KRT19, MUC1, EpCAM, CEACAM5, and BIRC5, resulted in higher detection rates compared to when each of the markers was used separately. In contrast to extracting mRNA from the PBMC pellet following centrifugation, as in previous studies, the multimarker detection was applied to cells isolated by immunomagnetic enrichment with anti-EpCAM and anti-MUC1 [34]. A similar approach was adopted by another group using a different gene panel consisting of human telomerase reverse transcriptase (h-TERT), CK20, CEA, and c-MET [57].

Genomic methods have also been combined with immunocytochemistry in order to increase the power of CTC detection and characterization. Immune staining with

CK, CD45, and DAPI was performed in addition to fluorescence in situ hybridization (FISH) with the centromere of chromosome 8 (CEP8) probe, following negative depletion enrichment for CTCs from blood of patients with benign and malignant pancreatic lesions. This approach utilized ploidy as marker of malignancy and showed that CTCs can be either CK positive diploid or hyperdiploid cells or CK negative hyperdiploid cells [37].

Alternative Detection Methods

Flow cytometry has been used to detect CTCs following density gradient centrifugation and staining of the isolated PBMC layer with antibodies against α5β4-Integrin, MUC-1, EpCAM, CD45, and also Hoechst and Propidium Iodide (PI) for nuclei and dead cell staining, respectively. Tumor cells were identified as α5β4-Integrin^{+}/EpCAM^{+}/Hoechst^{+}/CD45^{-}/PI^{-} cells by applying the relevant gating parameters [58]. Aptamers have also been used as probes for CTC detection. Aptamers are single stranded nucleic acid fragments, which specifically bind to a given molecule, even if the exact composition of that molecule is not known. In a method known as systematic evolution of ligands by exponential enrichment (SELEX), these fragments are developed by repeated exposure of the target molecule to a random nucleic acid library and selecting the sequence that binds to the target with the highest affinity. SELEX Aptamers that specifically bind to tumor cell constituents have been identified and have been used to detect pancreatic CTCs in patients' blood, showing similar efficacy to immunocytochemistry [59]. Another detection method has been developed to exploit the telomerase activity of cancer cells. In this case a recombinant telomerase-specific adenovirus, with a telomerase promoter at the 5-end of the viral genome and green fluorescent protein (GFP) at the 3-end, specifically infected CTCs and allowed their detection by GFP monitoring. This method was successful in detecting CTCs in a study where patients with various malignancies, including pancreatic cancer, were enrolled [60].

Finally, pancreatic CTCs can be detected by classic cytopathology using Giemsa or toluidine blue staining, following microfluidic filtration enrichment [39], or immunohistochemistry with EpCAM and CK following staining with hematoxylin and eosin [41].

Downstream Analysis Beyond Confirmation of Tumor Origin

Once cells have been detected, isolated, and confirmed as CTCs, they are released from the device and are ready for further downstream analysis.

Downstream analysis of pancreas cancer CTCs has been performed in a number of studies. Firstly, mutational analysis of the KRAS gene for codon 12 [40] and codon 13 [40] mutations from single CTCs has been successful with PCR followed by gel electrophoresis and Sanger sequencing. Another group performed whole genome amplification followed by copy number analysis with array comparative genomic hybridization and next-generation sequencing for the genes KRAS, TP53,

and NOTCH1, on CTCs isolated from spike in experiments with a PDA cell line [50]. mRNA microarray analysis has been used in a study trying to identify genetic signatures predictive of response [61]. Furthermore, by subjecting CTCs to single molecule RNA sequencing, a digital gene expression profile was derived which showed that the WNT gene family members were enriched in CTCs with increased metastatic potential, implicating WNT signaling in the metastatic process [62]. Whole genome microarray analysis on RNA extracted from CTCs has also been reported in a study which identified a gene panel consisting of nine genes involved in cell migration, motility, and invasion. The expression of this "cell motility gene signature" was enriched in CTCs [63].

Apart from genomic analysis, pancreatic CTCs were also subjected to molecular characterization with immunocytochemistry. A study used a panel of five markers, namely EpCAM, panCK, Vimentin, CK 7, and E-Cadherin, to characterize CTCs captured by ISET [41]. Finally, viable CTCs isolated by microfluidic approaches were successfully cultured in vitro [47].

Clinical Utility of CTCs in Pancreatic Cancer

Assessment of a suspicious pancreatic mass begins with a pancreatic protocol computed tomography (CT) scan. CT has a sensitivity of 90% and specificity of 99% in diagnosis of pancreatic cancer and the images correlate well with operative findings. Magnetic resonance imaging (MRI) and positron-emission tomography (PET) scans can also be utilized to assist with the assessment of difficult lesions and tumor resectability. However, imaging is not efficient in detecting early postoperative relapse. One confounding element is the associated profuse desmoplasia which, along with the postoperative inflammatory reaction, poses difficulty in identification of small recurring tumors. Moreover, despite the high sensitivity and specificity of imaging modalities, definitive diagnosis is obtained only by endoscopic ultrasound-guided fine needle aspiration (EUS-FNA). On average four passes are required to obtain adequate tissue for subsequent diagnosis. This method is characterized by high sensitivity and specificity, but the presence of chronic pancreatitis, which often accompanies pancreatic adenocarcinoma, can reduce sensitivity. In this case more passes are required [64], increasing the risk of complications. Unfortunately, this technique only provides a small piece of the primary tumor for histological examination and may not accommodate spatial tumor heterogeneity. Furthermore, EUS-FNA is very rarely performed for metastatic lesions, and moreover, serial biopsy is impractical precluding analysis of tumor evolution and dynamic changes before, during, and after treatment that could give insight into the development of drug resistance and provide targets for new therapeutic approaches.

With regards to less invasive biomarkers, there is a relative paucity in the field of pancreatic cancer. CA 19-9 is the most widely used serum biomarker in clinic. However, there are several factors limiting its clinical use. Firstly, 5–10% of the

population are Lewis blood group negative and therefore do not express CA 19-9 even in the presence of advanced pancreatic cancer. Furthermore, CA 19-9 levels are elevated in nonmalignant pancreatic and extrapancreatic diseases, giving false positive results on the one hand, and also being unable to differentiate between benign and malignant pancreatic disease on the other. CA 19-9 is also characterized by an extremely low positive predictive value of only 0.5–0.9%, being ineffective as a screening tool even in symptomatic patients [65].

It is therefore undeniable that better, additional biomarkers are required in many aspects of pancreatic cancer management, from screening, diagnosis, and staging to prediction of relapse and identification of resistance to treatment. Predictive biomarkers are required to stratify patients for personalized therapy and pharmacodynamics biomarkers are vital to support drug development. CTCs in which DNA, RNA, and proteins can be assessed have the potential to provide useful tools in the management of pancreatic cancer and CTC cultures for real-time therapy testing would be a step change for drug development and therapy selection. Table 1 summarizes studies where CTC-based analyses have been used to potentially aid clinical decision making such as for prognosis or response monitoring.

The Potential for CTC-Based Screening

No screening method is currently available for the early diagnosis of pancreatic adenocarcinoma, even for high-risk groups such as individuals with family history of this disease. Screening could lead to earlier diagnosis and consequently increase the number of cases for which surgery and adjuvant chemotherapy with curative intent is possible. A screening method should ideally be minimally invasive and of low cost in order to be suitable for large-scale application. A high positive predictive value for pancreatic malignancy is also necessary. So far, the combination of imaging with serum markers and genetic tests has not proven an effective screening method in familial pancreatic cancer [66].

CTCs have several characteristics that may prove useful in designing a screening tool. Firstly, circulating epithelial cells of pancreatic origin were detected in patients with precancerous pancreatic conditions such as intraductal papillary neoplasm or mucinous cystic neoplasm [67]. This finding supports the theory that epithelial cells circulate in the bloodstream before overt cancer development, providing evidence that findings from genetically engineered mice may also apply to humans. Secondly, circulating cells in patients with precancerous conditions are morphologically similar to those found in the circulation of patients with pancreatic adenocarcinoma and to cells from the primary tumor [39]. Finally, in a study, CTCs and/or CA 19-9 were positive in all patients with confirmed PDA, as CTCs were detected even in patients with normal CA 19-9 levels [36].

No large-scale clinical trial has been designed so far to specifically assess the robustness and cost efficiency of CTCs as a screening tool for pancreatic cancer. The evidence, however, suggests that this minimally invasive method may be useful in

Table 1 Potential clinical utility of circulating tumor cells in pancreatic Cancer

Clinical utility	Method	Patient number	Stage	CTC number	CTC cutoff	CTM	Ref.
Prognosis	ISET	60	Resectable	Mean: 2–4/ml depending on markers used	≥1	N/A	Poruk et al.
	CellSearch	79	Locally advanced	Median: 1/7.5 ml	≥1	N/A	Bidard et al.
	Density gradient centrifugation/ immunocytochemistry with panCK (AE1/AE3)	171	Stage I–IV	Median: $0.4/10^6$ cells	N/A	No	Z'graggen et al.
	Real-time RT PCR for EpCAM mRNA in cell lysate	58	Stage I–IV	N/A	N/A	N/A	Sergeant et al.
	Anti-EpCAM-conjugated supported lipid bilayer-coated microfluidic chip	63	Stage I–IV	Mean: 70.2/2 ml	≥70/2 ml	Mean: 29.5/ 2 ml Cutoff: ≥30/ 2 ml	Chang et al.
	ScreenCell	105 (PDA)	Stage I–IV	Not stated	≥1	N/A	Cauley et al.
	CellSearch and ISET	54	Metastatic-inoperable	Median: 0 (CellSearch) 9 (ISET)	≥1 CellSearch N/A ISET	Not detected (CellSearch) Detected (ISET)	Khoja et al.
Prediction of response	CAM-cell invasion assay	50	Locally advanced/ metastatic	Not stated	N/A	N/A	Yu et al.
Response monitoring	CTC-chip	15 (pancreatic)	Metastatic	Mean: 196 ± 228/ ml	N/A	N/A	Nagrath et al.
	GEM-chip	Not stated	Stage IV	Mean: 3/ml	N/A	N/A	Sheng et al.
	Immunomagnetic depletion of $CD45^+$ cells followed by immunofluorescence	41	Stage III–IV	Mean: 16.8 ± 16/ 7.5 ml	N/A	N/A	Ren et al.

this setting, especially for high-risk groups and for patients already diagnosed with preneoplastic conditions, though confirmation at the gene level that the circulating cells are confirmed as tumor cells is required to reduce false positive data.

CTCs for Pancreatic Cancer Prognosis

Identification of prognostic biomarkers is critical in both resectable and locally advanced/metastatic pancreatic cancer. Robust prognostic biomarkers would inform treatment decisions and guide management. In the case of resectable tumors, decisions regarding use of neoadjuvant or adjuvant chemotherapy could be based on prognostic biomarkers indicating worse prognosis. Thus only patients in the worst prognostic group will receive multimodality treatment, saving patients in the favorable prognostic groups from the side effects of chemotherapy. Prognostic biomarkers could also inform more intense follow-up of patients at high risk of relapse, to ensure earlier detection. In the locally advanced/metastatic setting, treatment strategies could be improved by selecting more aggressive chemotherapy regimens for patients with worse prognostic features. The use of CTC count as prognostic biomarker has been proven beneficial in metastatic breast, colorectal, and prostate cancer where the CellSearch assay ($EpCAM^+$/$Cytokeratin^+$ CTCs) is FDA approved. However, the data on CTC count in PDA are contradictory both in resectable and nonresectable metastatic tumors.

CTCs in Localized Disease

Initial evidence in localized disease using density gradient centrifugation to separate the mononuclear cell layer and immunocytochemistry to assign AE1/AE3 positive cells as CTCs showed that CTC number at baseline was not predictive of overall survival (OS). Interestingly, more advanced stage was significantly associated both with the presence of CTCs and worse survival, but this was not translated to a similar effect of CTCs on survival [42]. Also, in a study of 48 patients with PDA (40 resectable, 8 unresectable) where RT-PCR was used for the detection of EpCAM mRNA as a surrogate for CTC presence, there was no impact of the presence of CTCs pre- or postoperatively on disease-free survival (DFS) in the group of patients with resectable disease ($p = 0.28$). Of note, there is significant increase in EpCAM mRNA counts immediately after tumor resection ($p = 0.001$), indicating that surgical maneuvers may result in dissemination of cancer cells from the primary site. However, this phenomenon does not influence DFS [55]. A third study using the ScreenCell platform to filter blood from patients with pancreatic lesions demonstrated no significant difference in OS ($p = 0.69$) or time to recurrence ($p = 0.51$) between CTC-positive and CTC-negative patients in the PDA cohort ($n = 105$ of which 77 had resectable PDA). In this study, the presence or absence of CTCs did not correlate with disease resectability [39].

On the other hand, there are clinical studies to support CTC utility. Firstly, in a study of 67 patients with resectable biliary-pancreatic cancer (of which 34 had pancreatic cancer), the presence of CTCs as indicated by CEA mRNA positivity in

the nucleated cell layer following density gradient centrifugation of blood samples obtained during surgical resection predicted the risk of developing liver metastases with significantly higher risk ($p = 0.01$) in CTC-positive patients. This was translated into significantly worse OS ($p = 0.03$) in earlier stages (stage I–III). CTC detection rate was increased in blood obtained after tumor resection compared to blood obtained at the beginning of the operation, further supporting the phenomenon of cancer cell dissemination during surgery [43]. Furthermore, baseline CTC presence prior to operation was found to be a negative prognostic factor for OS ($p = 0.05$) in a large cohort of 172 patients undergoing surgery. In this study CTC presence was evaluated by RT-PCR for cytokeratin 20 mRNA again in the mononuclear cell layer of patient blood following density gradient centrifugation. Poor tumor differentiation was also associated with worse survival. However, this study showed that based on CTC presence, well and moderately differentiated (grade I and II) tumors could be subdivided in groups of better versus less favorable prognosis, potentially informing different management of these tumors [44].

More recent studies have gone beyond simple detection of CTCs, to CTC enumeration. Negative enrichment by CD45+ cell depletion followed by a combination of immunostaining with CK, CD45, and FISH with the centromere of chromosome 8 (CEP8) probe for CTC identification was used in one of such studies, where 61 patients with pancreatic lesions including 22 pancreatic cancer patients were analyzed. CTC-positive patients demonstrated worse survival rate ($p = 0.0458$) [37]. Also, another study using the same method in 25 patients with PDA (stages I–IV) showed that by using a cutoff count of 3 CTCs/7.5 mls of blood, patients with <3CTC/7.5 ml had significantly increased OS compared to those with >3 CTCs/7.5 ml (15.2 vs 10.2 months, $p = 0.023$) [36].

CTCs are not phenotypically homogeneous. In one study of 60 patients with PDA utilizing the ISET platform, a subset of CTCs with tumor-initiating capacity was significantly correlated with worse DFS ($p \leq 0.03$) and OS ($p \leq 0.01$). These CTCs express at least one of the tumor-initiating cell markers, namely CD133, CD44, and ALDH. In the same study, cytokeratin-only expressing CTC positivity was not significantly associated with survival outcomes [38]. Also in another study of 21 patients with PDA exploiting the ScreenCell platform, while the presence or absence of CTCs was not prognostic for survival, patients with CTCs harboring the KRAS mutation demonstrated better median OS ($p = 0.015$) [40]. These results stress the likelihood that the presence of specific CTC subpopulations may serve as better prognostic biomarkers compared to the whole CTC population.

Differences in CTC detection methods and insufficient statistical power are likely culprits in the discrepant data on CTC number and prognosis so far, along with a lack of consistency in patient selection and inadequate patient numbers for a statistically powered analysis. CTC heterogeneity is possibly another reason for the lack of clarity on CTC number for prognosis. A standardization of CTC enumeration with CTC molecular characterization in future adequately powered studies would lead towards better understanding of the prognostic role of CTCs and eventually may establish this parameter as a useful prognostic biomarker.

CTCs in Locally Advanced/Metastatic Disease

Studies that included patient groups with both localized, resectable and advanced, unresectable pancreatic cancer have reported a trend that CTCs are detected more frequently and in larger numbers in the latter group [41], [55], and [68], although this difference did not reach statistical significance. CTCs expressing mesenchymal markers (ZEB1) are more frequently detected in the metastatic setting ($p = 0.05$), possibly implicating this subgroup of cells to the development of metastatic disease [40]. However, the evidence so far has failed to demonstrate consistent results regarding the prognostic role of CTCs.

In the ancillary CircCe 07 study of the locally advanced pancreas cancer (LAP)-07 trial in 79 patients with locally advanced PDA, the presence of CTCs as assessed by the CellSearch platform at baseline and following 2 months of treatment did not correlate with PFS. However, CTC-positive patients at any time point had a significantly worse OS compared to CTC-negative patients ($p = 0.01$) [32]. Another study using a multimarker assay (RT-PCR for KRT19, MUC1, EPCAM, CEACAM5, and BIRC5 genes) for the detection of baseline CTCs following immunomagnetic enrichment in a cohort of 34 patients showed that the presence of CTCs was significantly associated with worse PFS ($p = 0.01$) [34]. Based on these results, it could be speculated that by increasing the efficiency of detection of CTC subpopulations using multiple markers, the latter study better captured tumor heterogeneity and derived a more accurate assessment of the prognostic role of CTCs. Interestingly, both of the above studies reported significant correlation between the presence of CTCs and tumor grade, with increased CTC positivity rate in poorly differentiated tumors. Finally, a study using two different methods for the detection and isolation of CTCs (CellSearch vs ISET) in 54 patients reported no significant difference both in PFS and OS between CTC-positive and CTC-negative patients, by either method [41].

The negative prognostic role of CTCs on survival was shown by three additional studies using different enrichment and detection approaches, that is, CellSearch [68], [69] and combination of CK, CD45 immunostaining with FISH for CEP8 following negative enrichment [37]. The presence of CTCs at baseline resulted in worse OS [37], [68], and [69]. Of note, CTC-positive patients also had increased serum levels of CA 19-9, but CTC positivity could predict worse survival outcomes even in CA19-9-negative patients, suggesting that combination of these two markers may have some value for prognosis [37]. Lastly, the importance of separation of the whole population of CTCs into subgroups and assessment of the prognostic role of these was demonstrated by a recent trial in 50 patients using the CellSearch platform, where even though CTC number per se was not significantly correlated with survival, patients with CTCs expressing MUC-1 had worse OS ($p = 0.044$) [51].

The prognostic role of CTCs in pancreatic cancer was more comprehensively evaluated by a meta-analyses that included nine studies and a total of 623 patients. This review found that CTC-positive patients had worse PFS ($p < 0.001$) and OS ($p < 0.001$) compared to patients with no detectable CTCs, establishing the presence of CTCs as a useful prognostic biomarker in pancreatic cancer [70].

CTCs as a Monitoring Tool for Response to Treatment

Accurate treatment response monitoring is of great importance for the optimal management of pancreatic cancer as development of resistance and clinical progression is common and happens early in the clinical course of this disease. Precisely identifying the time point of treatment failure would inform timely changes in the management plan. Furthermore, obtaining information regarding the mechanism (s) of treatment resistance would provide the basis for development of new and more effective treatment regimens. The challenge of serial biopsies, the rapid clinical deterioration that patients usually experience, and the unrealistic goal of routinely obtaining tissue from metastatic sites at times of disease progression all point to the urgent need for minimally invasive monitoring approaches.

Liquid biopsies that can be repeated over time may hold the key to improved treatment response monitoring. CTC burden may have potential as a surrogate of tumor burden and/or to predict treatment failure. Molecular profiling of CTCs would provide information about mutations associated with development of resistance. At the same time, liquid biopsies could provide insight into tumor heterogeneity, as both the primary and metastatic sites are represented in the pool of CTCs.

There are some emergent and promising preclinical data that support this view. In a PDX mouse model of PDA, CTC counts effectively mirrored treatment responses. In this study, CTCs were isolated using an anti-EpCAM antibody coated microfluidic device and enumerated based on their electric impedance signatures. Mice were randomized to receive either placebo or a phosphatidylinositol-3 kinase (PI3K) inhibitor, BKM120, which inhibits one of the main RAS-mediated PI3K downstream pathways. The two cohorts demonstrated no significant difference in CTC counts at baseline ($p = 0.8081$). CTCs were significantly decreased in the mice receiving BKM120 ($p = 0.0207$), whereas there was no change in the control group. While there was no statistically significant correlation between CTC number and tumor size ($p = 0.0547$), the fold change in CTC count and fold change in tumor volume were significantly correlated ($p = 0.004$), indicating that CTCs may be an effective biomarker of response [54]. The positive association ($p = 0.03$) between percentage change in CTC number, as measured by the CTC-Chip, and percentage change in tumor burden was confirmed by a clinical study in a cohort of patients with various malignancies, including three patients with pancreatic cancer receiving chemotherapy. This study again failed to demonstrate any association between absolute CTC number and tumor size [46]. However, this association was shown in a third trial which assessed CTCs as response monitoring biomarker in a small cohort of three patients with metastatic pancreatic cancer using the geometrically enhanced mixing chip microfluidic device (GEM-Chip). Here, CTC number decreased in parallel to CT scan-based tumor volume decrease and was associated with tumor size [47]. Finally, in another study that included 41 patients with advanced pancreatic cancer, following one cycle of 5-fluorouracil (5-FU) chemotherapy, CTCs were found in fewer patients and in decreased numbers compared to pretreatment. In this study CTCs were identified by negative enrichment with $CD45^+$ cell depletion followed by immunofluorescent staining with anti-CK8/18 and

anti-CA19-9. Of note, 20% of the post-chemotherapy CTCs displayed apoptotic changes [53].

Despite the positive results outlined above, a more recent study using the CellSearch platform in a cohort of 40 patients with unresectable pancreatic cancer undergoing chemotherapy or chemoradiotherapy showed no correlation between change in CTC number and treatment response based on CT scan [71]. This study leads to two important conclusions: firstly that standardization of the methods used for the detection and quantification of CTCs is absolutely necessary to draw conclusions with cross-site independent validation; secondly, once the most appropriate method is identified, further clinical trials with larger patient numbers are required in order to obtain reliable results regarding the efficacy of CTCs for monitoring of treatment responses.

CTCs as a Source of Predictive Biomarkers

CTC analysis was a useful approach to develop predictive biomarkers in a study of 50 patients with advanced PDA, where genomic profiling of pancreatic CTCs categorized patients into three groups (sensitive, intermediate, resistant) with regards to chemotherapy response. CTCs were captured in a cell adhesion matrix (CAM) using a cell invasion assay and subsequently were subjected to mRNA microarray analyses. Median PFS was significantly prolonged in the sensitive group compared to the resistant group, while PFS in the intermediate group was between the two extremes ($p = 0.0001$). Also median OS was significantly better in the sensitive group compared to the resistant one ($p = 0.0249$). This study showed that treatment responses could be predicted based on genomic analysis of isolated CTCs [61]. These exciting data could provide the basis for a personalized approach in the treatment of pancreatic cancer.

CTCs in Portal Venous Blood

A number of studies have examined CTCs in blood obtained from the portal vein of pancreatic cancer patients. The portal vein is the major draining blood vessel of the pancreas, providing a link to the liver. Liver capillaries serve as a filter through which blood containing CTCs need to travel before reaching systemic circulation. However, CTCs could be trapped in the liver, which may explain why liver is the most common site of pancreatic metastases and also why CTCs are detected in peripheral blood of pancreatic cancer patients less frequently and in fewer numbers compared to patients with other malignancies [15], [32], [41]. The first attempt to detect CTCs in portal venous blood in the setting of pancreatic cancer was in 20 patients with resectable PDA, where blood was obtained from the systemic and portal circulation simultaneously at operation and analyzed by CellSearch. CTCs were detected in the portal venous (PV) blood of patients that had no detectable CTCs in the circulation (five patients) and one patient had CTCs only in the systemic circulation. Also in

patients with CTCs in both PV and peripheral blood, more CTCs were detected in the PV compared to peripheral blood. Despite that no correlation with OS or disease free survival (DFS) was identified, CTC positivity in PV blood was associated with significantly increased frequency of liver recurrences ($p = 0.038$) [72]. These findings were also confirmed by another group, which by comparing PV and peripheral vein blood obtained at operation and analyzed by a microfluidic chip in a cohort of 60 patients with peri-ampullary or pancreatic adenocarcinoma showed that portal venous blood transports CTCs more frequently ($p = 0.0098$) and in higher numbers ($p = 0.0002$). PV CTC count was once again significantly correlated with higher rate of liver metastases within 6 months after surgery ($p < 0.001$) [73]. Importantly, PV blood can be safely obtained not only at operation but also by EUS-FNA, as shown in a study, where no immediate or delayed complications were observed following acquisition of PV blood. CTCs were present in PV blood in sufficient numbers (mean = 111.8 cells/7.5 ml, SEM ±35.3) to allow downstream applications [74].

Interestingly, more recent evidence suggests that the portal vein represents an immune tolerant environment, which promotes the presence and activation of CTCs. In a study of 41 patients with resectable pancreatic lesions including 21 with PDA, myeloid-derived immunosuppressor cells (MDSC) counts were significantly correlated with CTC counts and KRAS mutant mRNA expression, indicating that CTCs were actively transcribing mutant genes ($p < 0.0001$) [75]. MDSC exist in the portal vein and induce immune tolerance, so that normal flora or food particles that are absorbed from the bowel do not cause allergy or autoimmune reactions. Therefore, increased MDSC numbers may facilitate immune evasion and contribute to viability of transcriptionally active tumor cells in the portal vein, which in turn may explain both the higher rate of detectable CTCs in PV blood and the higher incidence of liver metastasis in patients with pancreatic cancer.

Circulating Tumor Microemboli

Apart from traveling as single cells, tumor cells can be found in the bloodstream in association with other cancer cells or noncancerous cells. These groups of cells are termed CTC clusters or circulating tumor microemboli (CTM). Evidence from an experimental mouse model of breast cancer has shown that CTC clusters are detached from the primary tumor as oligoclonal cell groups and are neither formed in the circulation by cell aggregation nor are derived from proliferation of a single CTC. CTC clusters demonstrate greater ability to form metastasis and have shorter half-life in the circulation compared to single CTCs [76]. The above properties lead to the hypothesis that CTC clusters are possibly entrapped within the capillaries where they extravasate and initiate formation of metastatic deposits. Therefore, their presence in the circulation may signify worse patient outcomes. Indeed, in small cell lung cancer, it has been demonstrated that the presence of CTM at baseline is significantly associated with worse PFS (HR = 2.07, 95% CI: 1.21–3.54, $p = 0.008$) and OS (HR = 2.94, 95% CI: 1.67–5.19, $p < 0.001$) [77].

The presence and clinical significance of CTM were also investigated in pancreatic cancer. CTM were detectable by ISET but not CellSearch in a study were both platforms were compared. Cells within CTM were heterogeneous with regards to expression of epithelial and mesenchymal markers, as both cytokeratin (epithelial marker) positive and negative cells were detected and also there was heterogeneity of the expression of E-cadherin (epithelial) and Vimentin (mesenchymal) markers [41]. In a more recent study, the detection of CTM by an EpCAM coated microfluidic device was used to categorize patients of both early and advanced stage to favorable versus unfavorable groups, using a cutoff of >30 CTMs/2 mls of blood. Patients in the unfavorable group demonstrated significantly worse PFS (2.7 vs 12.1 months, $p < 0.0001$) and OS (6.4 vs 19.8 months, $p < 0.0001$). Importantly, when a cutoff of >70 CTCs/2 mls blood was used, no statistically significant difference in PFS and OS was seen between the favorable and unfavorable groups, showing that CTM probably are a better prognostic biomarker compared to single CTCs [78].

Circulating Tumor Cell Derived Explants (CDX)

As already outlined, the population of tumor cells that circulate in the bloodstream contain the subgroup of cells with tumor-initiating capacity that is responsible for the generation of metastasis. It is therefore implied that transplantation of CTCs in immunocompromised mice would lead to tumor formation and development of in vivo models that could potentially recapitulate the biology of the most aggressive tumor compartment. CTCs enriched from the blood of patients with small cell lung cancer were injected into the flanks of immunodeficient mice and formed tumors. These patient CTC-derived explant models (CDX) demonstrated similar morphological characteristics to the primary tumors when assessed by histopathology and immunohistochemistry. Genomic analysis by next-generation sequencing and copy number aberration (CNA) analysis of matched CDX and patient tumors showed that CDX preserved the genomic signature of the tumor of origin. Finally, when the in vivo models were treated with cisplatin-etoposide chemotherapy, responses were similar to those of the donor patients [79]. More recently, CDX were also developed from melanoma patient CTCs with a success rate of 13% (6 out of 47 attempts). Again, CDX shared common morphology and immunophenotype with the original tumor and also demonstrated common genetic characteristics as assessed by whole exome sequencing (WES), CNA and RNA sequencing, and similar response to treatment, providing a potentially useful clinical decision making tool [80].

However, CDX models were first developed from hematopoietic cell depleted blood from patients with metastatic breast cancer, following transplantation into the femoral medullar cavity of immunodeficient mice. In this case, metastatic bone, lung, and liver deposits of human breast tissue origin were reported in six mice out of 118 attempts. Only samples containing at least 1109 CTCs, as measured by CellSearch, led to mouse tumor development, and these samples were drawn from three different patients from a cohort of 110. By surface marker analysis with fluorescence activation cell sorting (FACS), it was shown that $CD44^{+}CD47^{+}MET^{+}$ CTCs possess metastasis-initiating

capacity and that the number of these cells correlates better with disease progression and OS compared to the number of the whole CTC population [24].

To date CDX models have not been reported for pancreatic cancer. The rarity of CTCs in pancreatic cancer patient blood may pose a barrier to the development of such models. It is clear, however, that CDX could serve as a platform to study the complex biology and test novel treatments that will aid at tackling this particularly aggressive disease.

ctDNA in Pancreatic Cancer

Cell-Free DNA (cfDNA) and Circulating Tumor DNA (ctDNA)

Cell-free DNA (cfDNA), was first described in 1948 [81] and, more recently, has been the subject of intense study and is now being used in a number of clinical settings. Most studies and this review focus on cfDNA released into the bloodstream and assessed in plasma or serum; however, cfDNA can be examined from other body fluids, including urine, cerebrospinal fluid, stool, saliva, uterine lavage, and pleural fluid. Although the origin and any potential function of cfDNA remains unknown, it is thought to be released by dying cells and, based on observations that most cfDNA fragments appear to be 170–200 base pairs (bp) inter-nucleosomal fragments, it is inferred that cfDNA is derived primarily from apoptotic cells [82]. The half-life of cfDNA has been estimated to range from 16 min to 2.6 h [83], [84], with removal of cfDNA from circulation mediated by the kidneys, liver, spleen, and/or circulating nucleases [85]. Healthy individuals have detectable cfDNA; however, a number of physiological and/or pathological conditions can alter cfDNA size and concentration [86].

For patients with cancer, some of their cfDNA is released by tumor cells to generate circulating tumor DNA (ctDNA) and can provide a snapshot of genetic changes in the tumor itself. The tumor component of cfDNA in cancer patients was firmly established through cfDNA genomic analysis which identified canonical oncogene mutations in TP53 in patients with bladder cancer [87] and KRAS in patients with colorectal [88] and pancreatic cancer [89]. In a study of pancreatic cancer patients, matched tumor and cfDNA sequencing established that in each patient there was a precise match between the sequences observed in plasma cfDNA and tumor [90]. Based on the observation that tumor DNA is often detectable in the cfDNA obtained from cancer patient blood, the term ctDNA is now often used for all oncology cfDNA studies even though some samples may not contain any detectable tumor DNA.

Cancer patients can have higher overall levels of cfDNA compared to healthy individuals, although the levels can overlap [91]. Correlations have been made between cfDNA levels and tumor size [92] as well as staging [93] and ctDNA is shorter than cfDNA from healthy tissue [94]. However, although increased cfDNA

concentration and differential size can indicate the presence of cancer, this is not widely accepted as sufficient for a definitive diagnosis.

The low levels of ctDNA present in cancer patient blood samples represent a major technological challenge which has been met by the development and application of highly sensitive PCR methods and the application of next-generation sequencing (NGS). Targeted, PCR-based mutation analyses yield high sensitivity and the ability to pick-up single mutant DNA molecules. Allele-specific PCR techniques, for detecting hot-spot mutations in ctDNA, have been used for more than a decade [95]. Technologies, such as the amplified refractory mutation system (ARMS) and competitive allele-specific TaqMan PCR (castPCR), report sensitivities as low as 0.01% in clinical samples [96], with one study reporting mutation detection of one tumor-derived copy of DNA in a background of 200,000 wild-type DNA molecules [97]. Digital PCR methods are now matching the older PCR/qPCR technologies for analytical sensitivities and can provide additional advantages. The basis for digital PCR is to separate and amplify single DNA molecules and provide a digital readout for each molecule amplified. The approaches to digital PCR are differentiated largely by the method of partitioning the DNA, either by microfluidic chambers (Fluidigm and OpenArray) or generation of microdroplets using water-in-oil emulsions (ddPCR using Bio-Rad and RainDance Technologies). These highly sensitive and quantitative methods are now used extensively to quantify ctDNA levels [98]. However, these PCR-based approaches are limited in their multiplexing capacity that is, they typically address a single locus or nucleotide and require prior knowledge of the molecular genetics of the tumor type or individual tumor.

Two main approaches are used for NGS analysis of clinical samples: (1) amplicon-based approach which involves PCR amplification of defined regions and subsequent NGS of the amplified product and (2) pull-down or hybrid capture where genome-wide NGS libraries are prepared and the targeted regions selected by hybridization pull-down [99], [100].

With amplicon-based NGS analysis, it is possible to target many kilobases, using dozens to hundreds of amplicons [101], [102]. While amplicon-based NGS has high sensitivity, it is limited by numbers of primers and inability to multiplex across multiple genes as well as their inability to detect complex alterations, such as chromosomal rearrangements. Hybrid capture-based approaches can target dozens to hundreds of kilobases and thus increase the genomic regions studied [99]. Until recently, the analytical sensitivity of NGS was limited by error rates generated while generating NGS libraries which typically has a background error rate of ~1%. This is particularly important for cfDNA analysis since the tumor fraction may be low, meaning tumor-specific mutations may be obscured by background error rate. Recently, a number of methodologies (lab-based and bioinformatics approaches) have been developed to lower the limit of detection in the NGS approaches. Using molecular barcoding, running multiple replicates, error suppression using bioinformatics, or a combination of all, it has been possible to reliably detect ctDNA allele fractions below 0.1% [101], [103].

Comprehensive, Untargeted Analysis of ctDNA

With the introduction of NGS, it is now possible to interrogate the ctDNA in a more comprehensive genome or exome-wide approach, although it typically requires higher ctDNA concentrations (typically a minimum of 5–10%). The advantage of more comprehensive analysis is that there is no requirement for prior knowledge of the genetic landscape of the tumor and can find de novo mutations, as well as scrutinize complex genome rearrangements. While this comprehensive approach is powerful, it is still time-consuming, more expensive, and requires much higher depth sequencing, but with the aid of improved bioinformatics approaches, reductions in the cost per genome advances are being made in this field. Whole exome sequencing (WES) was reported for ctDNA in longitudinal blood samples in 2013, identifying mutations associated with acquired drug resistance [100]. However, due to the limited analytical sensitivity and high costs, it is not used commonly. Whole genome sequencing (WGS) is especially informative, as it is not limited to known changes/mutations and can follow tumor evolution and heterogeneity during disease progression and selective pressures from cancer treatments. Genome-wide studies revealed a dynamic and complex mutational landscape in cancer, but also the utility of ctDNA and liquid biopsies in general in the field of personalized medicine and beyond [104], [105]. As well as analyzing chromosomal aberrations, focal amplifications, and gene rearrangements using high depth WGS, it is possible to analyze plasma ctDNA copy number changes using low depth WGS [106]. This approach reliably detects somatic copy number changes in plasma ctDNA, down to 5% in a fast and cost-effective way [106], [107].

Clinical Utility of ctDNA and ctDNA in Pancreatic Cancer

A number of studies have shown that patients with detectable ctDNA have worse survival outcomes than those without [108]. It has also been reported that ctDNA is a significantly better prognostic predictor than commonly used markers, such as CA 15-3 and CA-125 levels in breast and ovarian cancer, respectively [109], [110]. Schwaederle et al. analyzed ctDNA in patients with multiple cancer types using a 54-gene panel and found 58% of the cohort had detectable ctDNA, with 71% patients carrying at least one actionable mutation linked to a specific therapy [111]. In a study across a range of cancers including pancreatic cancer, Bettegowda et al. found that ctDNA was detected in 82% of patients with stage IV disease and 47% with stage I disease, showing its potential for noninvasive early diagnosis [93].

Although relatively few clinical studies have looked at ctDNA in pancreatic cancer, the initial data looks promising and indicate that ctDNA may be a valuable addition to the current pancreatic cancer blood-based biomarkers such as CA19-9 [112]. In a relatively small study of 14 patients with advanced pancreatic cancer, pretherapy ctDNA levels correlated with both PFS and OS, and longitudinal

changes in ctDNA levels corresponded both with radiological follow-up data and CA19-9 levels [113]. Since mutations in *KRAS* occur in 90% of primary pancreatic cancer tumors [114], *KRAS* has been widely examined in ctDNA from pancreatic cancer patients and the results show that detection of mutant *KRAS* indicates worse OS [68], [115]. Sausen et al. showed an overall detection rate for *KRAS* mutations in cfDNA of 43% and that detection of ctDNA after resection was able to predict clinical relapse and poor outcome (with recurrence) 6.5 months earlier than with traditional CT scans [116]. Droplet digital PCR, a highly sensitive methodology which can detect single molecules, was used to identify the presence of *KRAS* mutations in cfDNA from 105 patients enrolled for pancreatoduodenectomy and showed mutations were detectable in 31% of cases and associated with significantly poorer OS [117]. A similar chip-based digital PCR study of 50 patients with pancreatic cancer reported an overall detection rate for *KRAS* mutations in cfDNA of 35% [118]. More recently both NGS and digital PCR have been used to study cfDNA from patients with metastatic pancreatic cancer with digital PCR used to examine additional genes found to be frequently mutated in pancreatic cancer (BRCA2, EGFR, KDR, ERBB2) [119]. In addition to confirming the value of ctDNA for prognosis, this study also reported the use of ctDNA for measuring tumor response [119].

Given the difficulty in obtaining tumor biopsies from pancreatic cancer patients, the development of ctDNA based assays are highly likely to be beneficial by providing an alternative source for molecular analysis. As an anecdotal example, in a patient with PDA, an *EGFR* deletion was detected in ctDNA, 7 months prior to confirmation using a matched tissue biopsy [120].

A recent large-scale study has found that selecting therapies based on genomic analysis could improve outcomes for patients with cancer, although given the limits of drug accessibility and availability, the study found only 7% of patients benefitted from the screening. The authors did state that further randomized trials were needed and by expanding the access to drugs, more patients could have benefitted in their trial [121]. Personalized therapies, selected based on molecular tumor data, are not currently conducted as part of routine clinical management of pancreatic cancer. Here, ctDNA clearly holds promise as a predictive and monitoring biomarker, but with limited current utility while there remains a paucity of effective treatments. ctDNA could, however, be usefully employed in future early clinical trials of targeted therapeutics for stratification and therapy response monitoring.

Conclusion

The poor outcomes achieved in pancreatic cancer, where conventional chemotherapy remains the basis for treatment, highlight the need for improved understanding of this disease. Easily accessible circulating biomarkers are a promising means of expanding our basic knowledge of pancreatic cancer with the goal that this

knowledge will translate into improved patient outcomes. The examination of both CTCs and ctDNA in pancreatic cancer has fallen behind their use in other cancer types such as breast, lung, prostate, and colorectal cancer. This most likely reflects the complex biology of pancreatic cancer as well as the technical and biological limitations of identifying CTCs and ctDNA in a small blood sample. For pancreatic cancer CTC analysis there is the promise of platform optimization incorporating pancreatic cancer-specific or EMT markers alongside improvements in isolation and analysis of CTM as well as the development of CDX models that can provide informative biological models. Furthermore, the analysis of CTCs present in the portal vein blood may provide greater understanding of metastatic dissemination to the liver, the most common metastatic site in this disease.

Similarly, the ability to detect and quantify ctDNA as well as define the presence of disease-specific and/or targetable mutations holds great promise for the treatment of pancreatic cancer. The correlation between ctDNA concentration and tumor size, staging, and survival outcomes combined with the fact that *KRAS* mutation is detected in >90% of PDA provides a target for allele-specific mutational analysis that could indicate the pancreatic tumor origin of ctDNA. Furthermore, temporal evaluation of ctDNA has the potential to monitor dynamic changes in both tumor burden and clinical course of the disease, earlier and more frequently than conventional imaging allows. In addition, improved interrogation of ctDNA by untargeted analysis can identify complex genomic aberrations and assist in defining the genomic landscape of PDA more comprehensively, ultimately providing novel targeted agents or improved patient management.

Ultimately, the clinical utility of blood borne biomarkers will be established through incorporation of the appropriate biomarkers into relevant clinical trials. For this to be successful and benefit pancreatic cancer patients, it is essential that the continued advances in CTC and ctDNA research are incorporated alongside any improved therapies in pancreatic cancer.

Cross-References

- ▶ Cancer Exosomes for Early Pancreatic Cancer Diagnosis and Role in Metastasis
- ▶ Development of Novel Diagnostic Pancreatic Tumor Biomarkers
- ▶ Development of Novel Therapeutic Response Biomarkers
- ▶ Differential Therapy Based on Tumor Heterogeneity in Pancreatic Cancer
- ▶ Emerging Therapeutic Targets in Pancreatic Adenocarcinoma
- ▶ Epigenetic Pharmacology
- ▶ Mouse Models of Pancreatic Exocrine Cancer
- ▶ Pancreatic Cancer Stem Cells
- ▶ Precision Medicine Based on Next-Generation Sequencing and Master Controllers
- ▶ Role of Tumor-Stromal Interactions in Pancreatic Cancer Invasion and Metastases

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Cancer Exosomes for Early Pancreatic Cancer Diagnosis and Role in Metastasis

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Contents

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is a treatment-recalcitrant and highly metastatic cancer. Recent studies have demonstrated that PDAC is associated with an increased release of small vesicles called exosomes that are ~40 to 130 nanometers in diameter. These exosomes may derive from pancreatic cancer cells, cancer-associated fibroblasts, and infiltrating immune and inflammatory cells. They carry a cargo rich in proteins, lipids, DNA, and microRNAs. Exosomes can modulate the tumor microenvironment, promote pancreatic cancer cell proliferation, invasion, and metastasis, and prime the pre-metastatic niche to facilitate formation of distant metastatic lesions. Components of the exosomal cargo may also serve as diagnostic biomarkers and guide the design of precision medicine strategies. Finally, exosomes have been proposed to act as biological nanoparticles that can be loaded with drugs for therapeutic use.

Keywords

Early diagnosis · MicroRNAs · Exosomes · Metastasis

Exosomes

Unique Origin of Exosomes

Exosomes are ~40 to 130 nanometer (nM) particles of endosomal origin that derive from multivesicular bodies (MVBs) from which they are released following MVB fusion with the cell membrane [1, 2], as shown in Fig. 1. Consequently, they express

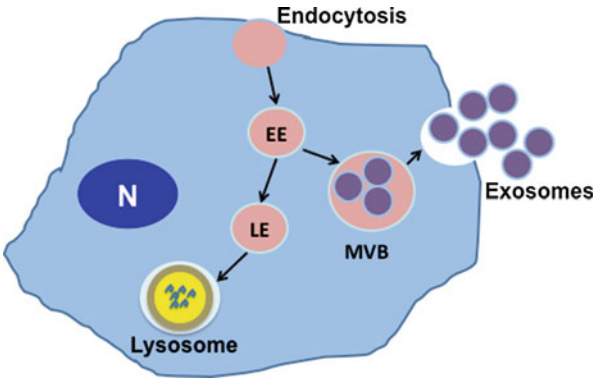

Fig. 1 Schematic representation of exosome formation. A cancer cell is shown exhibiting endocytosis followed by formation of an early endosome (EE) that can progress to become a late endosome (LE) or end up in a multivesicular body (MVB). Fusion of the MVB with the cell membrane leads to the release of exosomes. Most late endosomes end up in lysosomes where they undergo degradation. N: nucleus

endosomal proteins, including tumor susceptibility gene 101 (Tsg101) and Alix [1, 2], as well as many other proteins. By contrast to exosomes, microvesicles are shed directly from the cell membrane and are ~50 to 1000 nM in diameter, whereas apoptotic bodies are generated by the release of membrane blebs from cells undergoing apoptosis, and their diameter ranges in size form ~800 to 5000 nm [3–5].

It has been known for several decades that cells have the capacity to internalize fluids, large molecules, plasma membrane fragments, extracellular ligands, and cell-surface receptors through a process called endocytosis [6]. The internalized material ends up in multiple endocytic vesicles that undergo fusion to form the early endosome compartment. Some of this internalized material, including signaling receptors, can recycle from the early endosome to the cell surface, whereas other components are transported to the late endosomal pathway and subsequently to lysosomes where they undergo degradation [6].

Receptor-mediated endocytosis may occur through clathrin-coated pits on the cell membrane, and through clathrin-independent mechanisms, and is under complex regulatory control [7, 8]. Importantly, ligand-receptor dissociation occurs in the late endosome, whereas these complexes often remain intact in the early endosome and can continue to signal [9]. Since exosomes derive from MVBs that had formed from endosomes, exosomes also have the capacity to carry and deliver the internalized receptors to target cells where, in theory, they may participate in signaling events [9–11]. Conversely, growth factor receptor signaling can act to promote late endosome formation [12], suggesting that this compartmentalization mechanism serves to fine tune receptor-mediated signaling output.

Exosome Isolation Methods

Exosomes can be isolated from all bodily fluids, including blood, urine, and saliva. When seeking to study exosomes in the circulation, serum or plasma can be prepared by using red top or lavender top collection tubes, respectively. Lavender top collection tubes are coated with EDTA, which does not interfere with microRNA (miRNA) analysis.

Specimens should be promptly placed on ice or in a refrigerator (4 °C), taken to the lab within less than 60 min, and rapidly centrifuged (1000 x *g* for 10 min) at 4 °C. Supernatants can then be collected and stored at −80 °C until it is time to prepare the exosomes [13, 14], or subjected to a second optional centrifugation (10,000 x *g* for 10 min) at 4 °C to ensure the removal of any residual coarse debris prior to storage at −80 °C. To prepare exosomes, samples should be thawed on ice, centrifuged at 10,000 x *g* for 30 min (4 °C), and filtered through a 0.22 μm filter to remove remaining debris. Appropriate aliquots (for example, 250 μl/sample) can then be centrifuged at 110,000 x *g* for 2 h (4 °C). The resulting pellets should be washed with phosphate-buffered saline (PBS) to remove debris, and then resuspended in PBS prior to undergoing a second 110,000 x *g* centrifugation for 2 h (4 °C).

There are a variety of alternate methods for isolating exosomes [15–17]. A few examples include sucrose gradient fractionation, size-exclusion chromatography,

affinity chromatography, affinity immunoprecipitation, polymer-based precipitation as described in System Bioscience's protocol (https://www.systembio.com/downloads/Manual_ExoTC_WEB.pdf), and immunoaffinity capture [14, 15]. An example of the latter method is based on the observation that exosome extraction from solutions can be accomplished through their binding to bead-immobilized Tim4 via their surface phosphatidylserine (PS) [16]. Given that Tim4 binding to PS is Ca^{2+}-dependent, the captured exosomes can be released from the magnetic beads by Ca^{2+} chelation [16].

When studying exosomes released by cells during cell culture, it is important to remember that serum, including fetal bovine serum (FBS), contains exosomes and that these exosomes should be removed prior to use in cell culture studies. Alternatively, exosome-free FBS is commercially available.

Exosome Characteristics

Exosomes are vesicles consisting of a single membrane phospholipid bilayer with both surface and embedded proteins (Fig. 2). In addition to expressing endosomal proteins, exosomes are enriched for proteins deriving from cell membrane domains that tend to undergo internalization to form endosomes, such as tetraspanins, lipid-protein raft components, and adhesion molecules [17, 18]. Tetraspanins have four transmembrane domains and may be highly abundant in certain exosomes, depending on their cell of origin [3]. Exosomes are also rich in flotillins, which are involved in endocytosis [15], and annexins, which are phospholipid-binding proteins

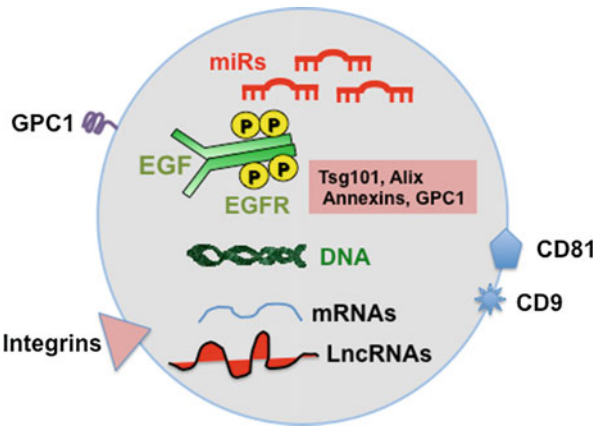

Fig. 2 Schematic representation of an exosome. The cholesterol-rich membrane of an exosome is shown decorated with the glycophosphatidylinositol-anchored glypican-1 (GPC1), the tetraspanins CD9 and CD81, and integrins. The cargo within the lipid bilayer of the exosome includes microRNAs (miRs), DNA, mRNAs, and long noncoding RNAs (LncRNAs). Cargo proteins include the EGF receptor (EGFR), TSG101, Alix, and GPC1, among many others

that are regulated by calcium and that have been implicated in the modulation of numerous cell processes including exocytosis [19].

Exosomes also tend to be enriched for proteins that are anchored to the cell membrane via a glycosylphosphatidylinositol (GPI) motif [20]. For example, the heparin sulfate proteoglycan (HSPG) glypican-1 (GPC1) is overexpressed in PDAC and is a GPI anchored protein [21] that contributes to PDAC angiogenesis and pancreatic cancer cell proliferation [22–24]. HSPGs are ubiquitous cell surface molecules consisting of core proteins covalently linked to glycosaminoglycans (GAGs) polysaccharide chains that are characterized by disaccharide repeats such as L-iduronic or D-glucuronic acid and either N-acetylgalactosamine or N-acetylglucosamine [25, 26]. GAGs may consist of heparin and heparan sulfate (HS), chondroitin or dermatan sulfates, as well as hyaluronan or keratan [26, 27]. Importantly HSPGs act as receptors that internalize exosomes [28] and HSPGs are also taken up by exosomes [28]. Therefore, it is not surprising that GPC1 was recently shown to be present in exosomes from patients with PDAC, normal control subjects, and patients with chronic pancreatitis [29]. The manifold components of the cargo of exosomes can be found in the ExoCarta database (http://www.exocarta.org).

Pancreatic Cancer and Exosomes and Diagnostic Potential

Size Distribution of Pancreatic Cancer Exosomes

Several techniques are generally used to assess the quality and size of the exosome preparation. Visualization by electron microscopy provides strong confirmatory evidence for the purity of the exosome preparation. However, alterations caused by sample fixation or dessication, or by exosome adherence to the template surface can alter the shape and apparent diameter of the exosomes [30]. Moreover, electron microscopy is both expensive and time consuming. Alternate techniques for assessing exosome size and number are generally used by many laboratories [31]. For example, the size distribution and concentration of exosomes in a biological fluid can be determined with readily available instruments. Thus, nanoparticle tracking analysis (NTA) allows for the measurement of particle size by determining the angular variation in intensity of scattered light following laser illumination [32]. NTA is readily performed in a reproducible manner by instruments manufactured by Nanosight [33]. By contrast, the qNano system uses a nanopore and tunable resistive pulse sensing to quantitate particle size and concentration [33].

Using the qNano system, a recent study evaluated the size of exosomes from normal controls, patients with PDAC, and patients with chronic pancreatitis [28]. The diameter of the majority of normal control exosomes ranged from 60 to 100 nm. By contrast, the diameter of CP and PDAC exosomes ranged from 70 to 120 nm [28]. However, only the PDAC-derived exosomes had numerous exosomes that ranged in size from 85 to 115 nm (Fig. 3a). Importantly, within 24 h following PDAC resection the diameter of the PDAC-derived exosomes in the circulation

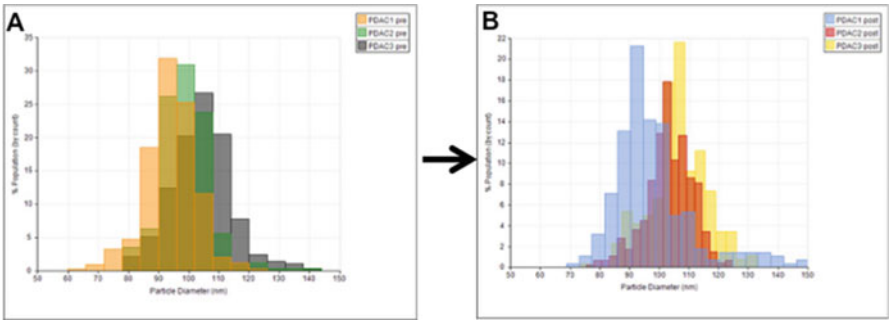

Fig. 3 Exosome distribution pre- and post-resection. Exosome diameter was determined using the qNano system. (**a**) Size distribution of exosomes prior to pancreatic cancer resection, when exosomes were mostly in the 85–115 nm range. (**b**) Size distribution of exosomes 24 h following PDAC resection was similar to that observed in control samples and in chronic pancreatitis samples. Data are from reference 28

reverted to the diameter in control samples (Fig. 3b). These observations suggest that high levels of 85–115 nm range exosomes point to the presence of an underlying PDAC and that these exosomes contain an altered, cancer-associated cargo.

Content of Pancreatic Cancer Exosomes and Diagnostic Utility

Studies with exosomes in pancreatic cancer patients have mostly relied on exosome isolation from serum or plasma, but PDAC-derived exosomes can also be found in saliva [34] and potentially other bodily fluids such as ascites, bile juice, and pancreatic juice. In the case of urinary exosomes, it has been demonstrated that following immunocapture on magnetic beads, it is possible to rapidly trypsinize the outer exosome proteins on the beads and identify them by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis [35].

Irrespective of their source, exosomes are stable in the circulation. However, in addition to their elimination into the urine, exosomes are also removed from the circulation due to their uptake by different cell types in various organs, as demonstrated in studies with miRNA-155-loaded exosomes that were injected into miRNA-155 knockout mice [36] and with fusion protein engineered for intra-exosomal expression and that had been labeled with radioactive iodine [37]. It is therefore likely that a steady state exists between exosome release into the circulation and exosome uptake in peripheral tissues and clearance by other means, which dictates the number and source of exosomes present in the blood. Nonetheless, the cargo within the exosome is protected from degradation, and this characteristic feature of exosomes enhances their diagnostic utility in disease states.

In addition to a complex intra-exosomal cargo that consists of proteins, miRNAs, long non-coding RNAs, mRNAs, transfer RNAs, lipids, and double-stranded DNAs that

have been shown to derive from all human chromosomes [5, 19, 35], PDAC-derived exosomes can carry mutated *KRAS* and *TP53* DNA, reflecting the mutation profile in the specific PDACs from which the exosomes were released [38]. In theory, therefore, it should be possible to establish signatures based on a mix of these biomarkers to confirm PDAC diagnosis and to monitor response to therapy, and ultimately to diagnose the disease at an early and resectable stage. For example, the combination of the levels of proteins, such as CD104, EpCAM, and Tspan8, together with the levels of miRNAs such as miRNA-1246, miRNA-4644, miRNA-3976, and miRNA-4306 has been reported to constitute a sensitive and specific signature for PDAC [39]. However, sensitivity and specificity issues remain to be addressed for many PDAC biomarker studies, even in relation to DNA mutation analysis [40].

Importantly, to date, early PDAC detection has remained an elusive goal [41]. Yet, it is widely accepted that noninvasive, informative biomarkers for early PDAC diagnosis are a major unmet need that have the potential to aid considerably in prolonging survival in this patient population. Although a well-executed recent study using an anti-GPC1 antibody reported that exosomal GPC1 may be diagnostic for early PDAC with high sensitivity and specificity [42], another study, using LC-MS/MS, revealed the presence of GPC1 in exosomes from normal controls and from patients with chronic pancreatitis, with overlap in exosomal GPC1 levels between the three groups [28]. The differences between the two studies could be due to the different methods used to detect this HSPG. Thus, one study used an anti-GPC1 antibody [42] and such an antibody could be directed against an aberrant glycan epitope that in theory could be cancer-specific. By contrast, the LC-MS/MS method used in the second study [28] measured the core protein and is not influenced by the glycanantion status of GPC1.

Exosomes from patients with PDAC carry high levels of miRNA-10b, miRNA-21, miRNA-30c, whereas the exosomal levels of all three miRNAs are low in normal control subjects or in patients with chronic pancreatitis [28]. Moreover, the elevated levels of all three miRNAs are greatly decreased at 24 h following PDAC resection, underscoring their PDAC origin [28]. It remains to be determined, however, whether a combined exosome and plasma signature could yield sensitive and specific biomarkers for early PDAC diagnosis and for monitoring PDAC recurrence following resection.

Given the clinical implications and potential benefits of early PDAC diagnosis, there has been a great deal of effort to devise advanced technologies to facilitate exosome analysis. For example, to enrich for PCC-derived microvesicles circulating in the plasma, Liang et al. designed a sensor chip that was coated with an antibody that targets the tetraspanin CD81, leading to the highly efficient capture and immobilization of microvesicles directly from plasma [43]. PCCs are believed to preferentially release exosomes (50–100 nm) over other microvesicles [44], so presumably, when using plasma from PDAC patients, this chip would mostly retain exosomes.

The captured vesicles were hybridized with gold nanospheres and nanorods that have been conjugated with antibodies against membrane-bound erythropoietin-producing hepatocellular receptor tyrosine kinase class A2 (EphA2), and anti-CD9,

respectively [43]. The use of gold nanoparticles provided two key advantages. First, the inertness of gold nanoparticles prevented spurious interactions. Second, gold nanoparticles are known to exhibit robust localized surface plasmon resonance, and the use of two different types of antibody-conjugated nanoparticles (nanospheres and nanorods) enhanced both the sensitivity and specificity of the assay due an increased intensity of the plasmon resonance and a readily detectable wavelength shift [43]. Using this nanoplasmon enhanced scattering (nPES) assay, Liang et al. showed that they can differentiate the high EphA2 vesicle signal from PDAC patients by comparison with corresponding signal from normal controls and chronic pancreatitis patients.

It will be important to confirm that specificity and sensitivity of the nPES assay will remain high as more samples are examined. It is also not clear whether this approach could be used to diagnose microscopic PDAC or early Stage IA disease. Finally, it should be noted that PDAC is rich in cancer-associated fibroblasts (CAFs), and these CAFs also release exosomes. It would therefore be interesting to determine whether the anti-CD81-coated sensor chip is also able to capture CAF-derived exosomes.

Pathological Actions of Exosomes in Pancreatic Cancer

Exosome Actions in the Tumor Microenvironment (TME)

Lung cancer cells release exosomes that carry miRNA-21 and miRNA-29a, and these exosomes bind Toll-like receptor 7 (TLR7) and TLR8 in immune cells, thereby eliciting an inflammatory response that enhances cancer cell proliferation and metastasis, revealing a novel role for exosomes in the TME [45]. Inasmuch as miRNA-21 is abundant in PDAC-derived exosomes [28], it is possible that a similar phenomenon may occur in the TME in PDAC. Additional potential actions in the TME include the modulation of oxidative phosphorylation or glycolysis by both cancer cell-derived and CAF-derived exosomes due, in part, to the inhibition mitochondrial oxidative phosphorylation and providing an energy supply to the cancer cells through the exosomal cargo of amino acids and lipids [46, 47].

It is possible that exosomes within the TME may also transfer to noncancerous cells mutant Kras protein and tyrosine kinase receptors such as the epidermal growth factor (EGF) receptor (EGFR) and ligands that bind to EGFR, such as amphiregulin, thereby creating a field effect that nurtures tumor growth [48–50]. In addition, through their stimulatory effects on the conversion of pancreatic stellate cells (PSCs) into CAFs, exosomes can act to enhance PDAC desmosplasia, and through their ability to induce EMT and inhibit cancer-directed immune pathways, exosomes can promote PDAC metastasis [51, 52]. Exosomes also contain proteases and can therefore degrade components of the extracellular matrix such as collagens, fibronectin, and laminins [53]. In turn, ECM degradation liberates growth factors and

matrix metalloproteases that combine to exert mitogenic, motogenic, and invasion promoting effects on the PCCs while also activating pro-survival pathways and apoptosis resistance in the PCCs.

PDAC-Associated Exosomopathies

PDAC may be associated with systemic prodromal manifestations that appear prior to the cancer diagnosis. Such prodromal syndromes include pancreatogenic diabetes, which is also known as type 3c diabetes mellitus or T3cDM diabetes [43, 54], unexplained weight loss, which could be viewed as a pre-cachexia state, and thromboembolic events that have also been described as Trousseau syndrome.

The mechanisms underlying T3cDM have not been completely delineated but include resistance to insulin actions. In addition, a recent study [43] reported that one mechanism for T3cDM is the release by the PCCs in PDAC of exosomes that carry adrenomedullin (AM). Following release into the systemic circulation these exosomes return to the pancreas, enter the islets, and interact with the β-cells where AM delivery induces an unfolded protein response that interferes with β-cell function and may even lead to β-cell death [43]. Consequently, these patients cannot mount a robust insulin response to their insulin-resistant state, and therefore exhibit rising blood glucose levels. Thus, T3cDM can be viewed as an exosomopathy [55].

In spite of a great deal of progress in understanding the multiple pathways that contribute to cancer cachexia [56], the potential role of exosomes in PDAC-associated cachexia is yet to be fully explored. Nonetheless, it is now recognized that microvesicles from PC1, Panc-2, and MIA PaCa 2 PCCs carrying miRNA-21 can signal through TLR7 in Pax7-positive murine myoblasts to activate c-Jun N-terminal kinase and promote muscle cell apoptosis [57]. By contrast, similar myoblasts prepared from $TLR7^{-/-}$ mice were resistant to apoptosis when exposed to either conditioned medium samples from Lewis lung carcinoma cells that induce cachexia in mice, or to serum samples from 5 of 7 pancreatic cancer patients who were diagnosed as having cancer cachexia [57]. Of note, Pax7 is a transcription factor expressed in the nuclei of muscle stem cells that controls their self-renewal. Previously, it was shown that its persistent expression in muscle stem cells during cachexia prevents them from differentiating into adult muscle cells and/or from fusing to damaged myofibers and thus impedes myofiber repair and promotes muscle atrophy [58]. It remains to be determined whether such a mechanism is active in relatively early stages of PDAC, whether TLR8 in humans mediates the same pathways as TLR7 to induce muscle stem cell apoptosis, and what ultimately dictates the fate of these Pax7-positive satellite cells between failure to differentiate vs. apoptosis.

With respect to the third type of exosomopathy, while the underlying etiologies in venous thromboembolic events in PDAC are not well understood, they have been correlated with elevated plasma Tissue Factor (TF) levels [59]. This correlation has

also been observed in a mouse xenograft model of PDAC [60]. TF is carried by microparticles [61], and therefore may also be carried by exosomes. However, in the case of melanoma cells, it was shown that most of the TF is found in microvesicles and apoptotic bodies [62]. Nonetheless, monocyte-derived exosomes have been shown to exert pro-thrombotic actions [63]. Given that thromboembolic events are an important cause of death in cancer patients receiving chemotherapy as outpatients [64], this aspect of PDAC pathobiology needs further exploration.

Exosomes and the Metastatic Niche

The metastatic process consists of a complex sequence of events that includes different types of cancer cell migration, EMT, invasion, immune alterations within the TME, and, systemically, extracellular matrix degradation, breaching of barriers in a manner that enables the cancer cells to enter into blood vessels and lymphatics, survival of these cells in the circulation, and successful colonization of distant organs within a receptive microenvironment called the metastatic niche [65–67]. Recent studies have highlighted the important role of miRNAs in cancer progression and metastasis due to their ability to regulate cell proliferation, migration, invasion, and metastasis [68–73].

Exosomes can promote cancer metastasis by carrying deleterious miRNA and proteases to distal sites where they prime the normal microenvironment to be receptive to circulating cancer cells [53, 74]. In addition, exosomes can target immune pathways in a manner that promotes the metastatic process. For example, exosomes have been reported to exert effects on the distant "soil" by priming the pre-metastatic niche to be receptive to metastatic cells. Exosomes were also suggested to increase the number of myeloid derived suppressor cells (MDSCs) in the TME which leads to the release of inflammatory cytokines that in turn prime the pre-metastatic niche [75].

Costa-Silva et al. performed a crucial study that definitively demonstrated an important role for PDAC-derived exosomes in priming the pre-metastatic niche in the liver [76]. They showed that purified exosomes from different murine models of PDAC (5 μg exosomes per injection) every other day for 3 weeks prime the hepatic pre-metastatic niche to be receptive to intrasplenic injections of PCCs, yielding macro-metastatic lesions 3 weeks later. Mechanistically, they demonstrated that the exosomes are taken up by the Kupffer cells and induce transforming growth factor β (TGF-β) expression that in turn upregulates fibronectin production by hepatic stellate cells [76]. The abundant fibronectin leads to the recruitment and retention of bone marrow–derived macrophages. Importantly, exosomal macrophage migration inhibitory factor (MIF) was required for TGF-β upregulation, and in the absence of MIF or following macrophage ablation, the exosomes no longer exerted a pro-metastatic effect. Clinically, exosomal MIF levels were higher in stage I PDAC patients that eventually developed hepatic metastases by comparison to stage I patients who did not develop such lesions. As pointed out in an accompanying commentary [77],

other exosomal components may also be important for the metastatic process both in terms of metastatic sites and in terms of PCC proliferation, which may also include unrecognized intrinsic characteristics of the PCCs. These findings are also in agreement with a long-standing observation that high levels of TGF-β isoforms in PDAC patients who had resectable disease and who did not receive any post-operative therapies were associated with earlier disease recurrence and shorter overall survival by comparison with patients whose PDAC expressed low levels of these isoforms [78]. It may therefore be timely to reconsider the reluctance to target TGF-β in clinical trials in patients with PDAC.

Therapeutic Implications

Exosomes for Drug Delivery

Initial efforts at using exosomes as drug delivery "nanoparticles" began in the past several years. For example, Alvarez-Erviti et al. reported that it is possible to deliver short interfering RNAs (siRNAs) to the mouse brain by systemic injection of targeted exosomes, based on the fact that exosomes cross the blood-brain barrier [79]. The exosomes were self-derived from dendritic cells, thereby avoiding any possible immune reactions against foreign exosomes, and were targeted to the central nervous system by engineering a fusion of lamp2b to neuron-specific RVG peptide [79].

In a subsequent study, it was shown that exosomes can be targeted to breast cancer cells expressing high EGFR levels by engineering the cells to express the GE11 peptide that is known to bind to EGFR, and making sure that expression is directed to the cell membrane by using a vector expressing the transmembrane domain of the platelet-derived growth factor receptor [80]. The authors then showed that the intravenous injection of exosomes from the engineered cells can deliver miRNA let-7a to breast cancer xenografts [80]. A variety of other strategies have been proposed to improve exosomes as drug delivery vehicles [81], and a more recent study demonstrated that it is possible to engineer exosomes to express a single domain antibody (nanobody) against EGFR using a GPI-based anchoring strategy, thereby greatly enhancing exosome delivery to EGFR overexpressing cells [82].

A novel therapeutic approach for PDAC was recently proposed based on the fact that oncogenic *KRAS* is the major truncal mutation in this malignancy and that the mutant Kras protein has been resistant to targeting. Using exosomes derived from normal fibroblast-like mesenchymal cells that were engineered to express either the siRNA or the hairpin RNA (shRNA) that specifically downregulate $Kras^{G12D}$, the most common type of mutated *KRAS* in PDAC, Kamerkar et al. demonstrated dramatic efficacy in several mouse models of PDAC [83]. It seems likely that this strategy will also be successful with other forms of mutant Kras, but this remains to be demonstrated.

Conclusion

It is likely that following up on the advances of recent years, it will be possible to develop novel strategies for early PDAC diagnosis that are based on the analysis of exosome cargo and that this approach will also yield novel prognostic markers. Moreover, advances in DNA mutational analysis of exosomal DNA could guide novel precision medicine approaches and the rapid monitoring of therapeutic responses. Exosomes will also be increasingly useful as drug delivery vehicles and as positive modulators for activating cancer-directed immune mechanisms. The combination of these new tools will likely dramatically improve the survival of patients with PDAC.

Key Research Points

- Exosomes are important intracellular regulatory vesicles within their cells of origin.
- Exosomes are released into their microenvironment and biological fluids, where they regulate numerous cellular processes.
- In PDAC, exosomes may exert effects on the tumor microenvironment to enhance PDAC growth and metastasis and to prime the pre-metastatic niche.

Future Scientific Directions

- There is a need for improved and highly reproducible assays of exosome content.
- There is a need for strategies to promote beneficial effects of exosomes and to block their deleterious effects.
- There is a need to understand how to modify endogenous exosomes for therapeutic purposes.
- There is a need to gain a better understanding of the therapeutic applications of exogenous exosomes.

Clinical Implications

- Exosome cargo can be analyzed to establish diagnostic and/or prognostic signatures in PDAC.
- Exosomes can be used as therapeutic vehicles.

Specific medical conditions associated with PDAC such T3cDM, cachexia, and venous thromboembolic event may be aggravate by exosomes and targeting these exosomes could lead to improved survival and improved quality of life in patients with PDAC.

Cross-References

- ▶ Development of Novel Diagnostic Pancreatic Tumor Biomarkers
- ▶ Development of Novel Therapeutic Response Biomarkers
- ▶ Diagnostic Biomarkers
- ▶ Emerging Therapeutic Targets in Pancreatic Adenocarcinoma
- ▶ Paraneoplastic Syndromes in Pancreatic Cancer
- ▶ Precision Medicine Based on Next-Generation Sequencing and Master Controllers

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Metabolism in Pancreatic Cancer

Ioannis Poursaitidis and Richard F. Lamb

Contents

Abstract

Despite knowledge of an increasing number of genetic changes present in pancreatic ductal adenocarcinoma (PDAC), the most common form of pancreatic cancer, it remains one of the cancers with the poorest prognosis, and the development of novel therapies that target its unusual biology and metabolic features is imminently required. Pancreatic tumor cells are thought to evolve under the conditions of limited oxygen and nutrient supply due to high levels of stromally produced extracellular matrix and associated poor blood supply. The prevalence of oncogenic KRAS mutations in PDAC, together with inactivation of TP53,

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CDKN2A, and SMAD4, predicates the engagement of distinct adaptive metabolic features that maximize the uptake and utilization of limiting oxygen and nutrients. Rewiring of the metabolism of glucose, amino acids, and lipids provides biosynthetic/metabolic intermediates required to maintain proliferation and survival, while the induction of autophagy and macropinocytosis permits repurposing of nutrients by PDAC tumor cells. Finally, PDAC tumor cells affect their neighboring cells, activating pancreatic stellate cells to produce a dense fibrotic stroma and provide nutrients in a paracrine manner, while inhibiting an effective antitumor immune response by restriction of nutrients from immune effector cells. It is hoped that by targeting such aberrant metabolism and nutrient utilization additional therapeutic options might soon be available in PDAC.

Keywords
PDAC · Metabolism · KRAS · p53 · Hypoxia · HIF · Desmoplasia

Introduction

PDAC is among the cancer types with the poorest prognosis, with around roughly 367,000 new cases diagnosed and 359,000 deaths in 2015. Overall PDAC has an extremely poor 5-year survival rate of around 6–8% that has not satisfactorily improved over the last four decades, and thus PDAC is expected to become the second most common cause of cancer-associated death before 2030 [1].

PDAC is thought to initiate and progress from a population of microscopic premalignant lesions termed PanINs (Pancreatic Intraepithelial Neoplasias) through multiple stages in a process that may take over two decades, while remaining asymptomatic to the patient [2]. Currently, there are neither diagnostic symptoms nor robust tumor biomarkers that might easily reveal the development of PDAC over this timeframe [3]. Thus, PDAC tumor cells can disseminate, resulting in metastasis to distant sites prior to overt diagnosis [4]. The main oncogenic event in PDAC, identified in the late 1980s, is mutations in the protooncogene KRAS [5], which occur in more than 90% of PanIN and PDAC [2, 6]. In later stages of PDAC progression, mutations and deletions of tumor-suppressor genes such as TP53, CDKN2A, and SMAD4 also occur with variable frequency [7].

Despite mutations in the proto-oncogene KRAS being identified as the key PDAC tumor-driving oncogene and the most common genetic change, and evidence that it plays distinct roles in mouse models both in tumor initiation and tumor maintenance (reviewed in [8]), the development of direct inhibitors of mutant KRAS has been problematic. Other indirect approaches that act to prevent the translocation of KRAS to the plasma membrane, its activation by exchange factors, or act in a synthetic lethal manner, may be more promising [9]. Current treatments in PDAC however utilize instead relatively nonselective cytotoxic agents, often in combination, but unfortunately with limited efficacy [1]. Moreover, such treatments can be difficult to tolerate without robust responses in many cases [1]. Therefore, there is a clear clinical need

both to understand in more depth the mechanisms of PDAC tumor evolution and heterogeneity, and to understand mechanisms of tumor maintenance in the face of limiting nutrients in order to develop new therapeutic strategies.

Recent studies have unveiled many metabolic adaptations activated downstream of KRAS signaling, which play important roles in determining the unique biology characteristic of PDAC, and which may ultimately lead to novel therapeutic regimens that target metabolic addictions found in PDAC (reviewed in [10–12]). These adaptations are found associated with several unique features of the biology of PDAC. In particular, the pancreatic tumor microenvironment contains a dense fibrotic stroma termed desmoplasia and a relatively low cancer cell cellularity in which intratumor interstitial pressure is high relative to normal pancreas [13]. Aside from PDAC tumor cells themselves, PDAC tumors contain a large population of activated fibroblasts (termed pancreatic stellate cells, PaSCs, or PSCs), which normally reside in exocrine areas of the pancreas, and various immune cells within the tumor microenvironment [14]. The high interstitial pressure found in advanced PDAC is thought to result at least in part from extensive deposition of ECM proteins from PSCs, including the glycosaminoglycan hyaluronan which is a CD44 ligand involved in cell-ECM adhesion [13]. As a result, the vascular capillaries collapse, limiting perfusion of oxygen and nutrients to the tumor and generating a hypoxic tumor microenvironment [14, 15] that acts to impair drug delivery to tumor cells [13]. Despite these harsh environmental conditions however, PDAC cells are capable of surviving, proliferating, and metastasizing due to various metabolic adaptations.

Nutrient Sensing

In normal cells, the utilization of extracellular nutrients depends both on the metabolic needs of the cell and their detection by a variety of nutrient-sensing mechanisms that prepare the cell to utilize nutrients for anabolic or maintenance functions. These mechanisms are normally tightly regulated to recognize changes in nutrient availability and elicit differential responses [16]. Critical responses that have been well studied include the activation of Adenosine MonoPhosphate–activated protein Kinase (AMPK) upon glucose restriction, and inhibition of the mechanistic Target of Rapamycin Complex 1 (mTORC1) upon amino acid restriction.

AMPK is generally activated physiologically upon detection of decreased cellular ATP levels, resulting in AMP accumulation, and phosphorylates an increasing variety of substrates [17]. Overall the result of AMPK activation in most cells is a cessation of energy-consuming anabolic processes such as protein and lipid synthesis, an enhancement of glucose and lipid catabolism to regenerate ATP, or induction of autophagy [17]. mTORC1, on the other hand, is activated by the presence of both growth factors and amino acid nutrients, promoting various aspects of cell growth including ribosome biogenesis and inhibiting autophagy, and in normal cells is inhibited by energy stress or by overall or specific amino acid restriction [16].

AMPK can itself repress mTORC1 activation, either by phosphorylation and activation of the mTORC1 pathway inhibitor, tumor sclerosis complex 2 (TSC2, [18]) or by phosphorylation of Raptor [19], a regulatory and structural component of the mTORC1 complex. The activation of mTORC1 inhibits autophagy [20] and therefore AMPK can induce autophagy either directly or indirectly through negative regulation of mTORC1. Additionally, AMPK can itself directly activate both bulk autophagy and mitophagy (breakdown of mitochondria) independently of its regulation of mTORC1 through phosphorylation and activation of ULK1 (Unc-51 Like Autophagy Activating Kinase 1) the mammalian ortholog of Atg1, a key initiator of the autophagic process in yeast [21]. AMPK can further promote mitophagy directly by facilitating mitochondrial fission through phosphorylation of a fission-promoting protein present on the outer mitochondrial membrane, MFF (Mitochondrial Fission Factor [22]).

During tumor progression, tumor cells are now appreciated to undergo metabolic reprogramming which enables them to utilize anabolic and catabolic pathways in a manner that promotes their survival and unrestricted proliferation. PDAC tumor cells exhibit many such adaptations and are capable of surviving in hypoxic microenvironments as well as in metastatic niches by activating both nutrient scavenging and nutrient acquisition pathways [10]. These strategies endow PDAC tumor cells with a selective advantage over normal pancreatic cells and are thought to be critical to promote their sustained viability and proliferation under harsh environmental conditions [11]. However, these aspects of deregulated PDAC cell function may themselves represent tumor-specific vulnerabilities and be sensitive to targeted therapies [10, 12].

Autophagy

The PDAC tumor microenvironment is characterized by local hypoxia [23] and limited accessibility to nutrients [15]. Therefore, PDAC tumor cells utilize a number of scavenging mechanisms to exploit the limited nutrients available through the vasculature. One of these mechanisms, leading to nutrient recycling, is autophagy (also termed macroautophagy), a process that normally results in the regulated degradation and recycling of cellular components for biosynthesis [24]. However autophagy also performs an important function in normal cells in cellular quality control by acting to eliminate potentially toxic protein aggregates and/or damaged organelles [25].

Through autophagy, macromolecules are first sequestered within double-membrane microtubule-associated protein 1A/1B-light chain 3 (LC3)-positive vesicles, the autophagosomes. Through a regulated series of events, autophagosomes ultimately fuse with lysosomes forming autolysosomes that mediate the digestion of the internalized cytoplasmic components. The autolysosome digestion products, namely amino acids, nucleotides, fatty acids, sugars, and ATP, are then transported back from the lysosome to the cytoplasm where they serve as biosynthetic precursors, cofactors or as an energy source for cells undergoing nutrient

starvation [26]. One of the key mechanisms used by nutrient-deprived cells for autophagy initiation is via the activation and suppression of the protein kinases AMPK and mTORC1, which regulate autophagic capacity through ULK1/2 and ATG13 proteins [24]. An alternative mechanism of autophagic stimulus (that is independent of ULK1 activation) occurs through the accumulation of ammonia produced by amino acid catabolism during glucose restriction [27].

Previous studies have indicated critical, but contrasting, roles for autophagy in cancer, and the same appears true in PDAC [28]. On the one hand, autophagy is thought to constitute a barrier to tumor formation through mitigation of oxidative stress/ROS and subsequent effects upon genomic integrity within premalignant PanINs [29]. In contrast, autophagy can also promote tumor formation in a number of cancer model systems (reviewed in [28]). In the progression of PDAC, autophagy appears also to play opposing tumor suppressive and tumor promoting roles [30]. Evidence for tumor suppressive function(s) of autophagy comes from pancreas-specific knockouts of autophagy regulators Atg5 or Atg7 that show an augmented emergence of KRASG12D-driven premalignant pancreatic lesions following autophagy inhibition [31]. Thus, it appears that in the early stages of PDAC progression autophagy acts in a tumor suppressive manner to prevent the initiation of premalignant lesions that act as a precursor to PDAC.

However, it is also clear that elevated basal autophagy (in the absence of starvation of nutrients) is a major feature of PDAC tumor cells, even when such cells are grown ex vivo under cell culture conditions in which nutrients are unlikely to be limiting, and is itself required for tumor progression in vivo [31]. Induction of autophagy however appears to be a relatively late event in PDAC development [31] and increased autophagy, as determined by LC3 immunocytochemistry, correlates with poorer clinical outcome in PDAC patients [32]. At least in part this is likely to be due to autophagy inhibition leading to a reduced degree of tumor cell proliferation rather than survival and is known to be dependent in some contexts upon intact TP53, but not in others [10]. Using an inducible mouse model of mutated KRAS in a p53Lox/WT background, thought to be analogous to that occurring in advanced PDAC, has shed further light on the role autophagy performs in advanced PDAC development. Ablation of KRAS in this model results in pancreatic tumor regression within 2–3 weeks followed by relapse after a few months. Transcriptome analysis of tumor cells surviving KRAS ablation revealed a significant enrichment of genes involved in lysosomal activity, mitochondrial electron transport chain, and autophagy, indicating that induction of increased autophagy and lysosomal activity was critical for tumor relapse [33].

Major questions that have arisen following these observations are what mechanisms lead to increased basal autophagy, and how they are related to the specific genetic changes found in PDAC? The increased number of autophagosomes and lysosomes frequently identified in PDAC tumor cells [34] suggests that some deregulation of the regulatory mechanisms controlling the abundance of these vesicular organelles occurs in PDAC. Indeed some human PDAC cells have been shown to exhibit both increased expression, and loss of cytoplasmic retention, of members of the microphthalmia/transcription factor E (MiT/TFE) family of

transcription factors [34] that are known to induce a transcriptional program that acts to increase lysosome biogenesis and therefore lysosomal catabolism [35].

In normal cells cultured under nutrient replete conditions, MiT/TFE factors are thought to be negatively regulated via phosphorylation by mTORC1 present on lysosomal membranes, leading to their interaction with cytosolic 14-3-3 proteins and nuclear exclusion [35, 36]. However, in PDAC cells, and despite elevated mTORC1 activity, MiT/TFE factors appear to be preferentially nuclear [34], indicating that additional regulatory events active in PDAC cells act to override mTORC1-mediated regulation, thereby promoting nuclear localization of MiT/TFE factors. Although the nature of these mechanisms, and how they operate preferentially in PDAC tumor cells, remain to be elucidated, they may impact on the function of importins such as IPO8/7 that direct nuclear import of specific cargo [37]. Thus, knockdown of these two importins in PDAC cells has been shown to prevent MiT/TFE nuclear localization [34].

Autophagy as a tumor promoting process might also represents a therapeutic target in PDAC. The antimalarial drug chloroquine (or its analog hydroxychloroquine, HCQ) is thought to block autophagic flux by increasing the normally low pH typical of lysosomes, thereby blocking the final stage of autophagy [38]. In preclinical studies, HCQ treatment has shown promise in inhibiting tumor growth in patient-derived xenograft (PDX) and human PDAC cell line xenograft mouse models [39]. Currently HCQ is in fact under evaluation in several clinical trials for PDAC treatment in the US, including as a single agent in metastatic cancer (trial designation: NCT01273805), in combination with gemcitabine (NCT01128296, [40]), in combination with gemcitabine/nab-paclitaxel (NCT01506973), or in combination with capecitabine with either radiation or proton therapy (NCT01494155). However, a key shortcoming of HCQ treatment is its poor drug pharmacodynamics whereby relatively long periods of drug administration are required to reach therapeutic levels [41]. However, when HCQ has been used under conditions where evidence of autophagy inhibition has been clearly established as a biomarker of drug efficacy [40], improved disease-free and overall survival in PDAC patients has been demonstrated. As an alternative to HCQ, Lys05, a novel dimeric derivative of chloroquine, has been shown to have significant in vivo activity, both as a single agent [42] and in combination with a BRAF inhibitor [43]. Interestingly, the inhibition of autophagy in PDAC is known to incur additional effects on metabolism that may have therapeutic implications. Thus, upon autophagy inhibition PDAC cells have been found to utilize less oxygen during oxidative phosphorylation in mitochondria, and instead switch to increased dependence upon glycolysis as a source of ATP [28, 31].

Macropinocytosis

In addition to autophagy and the recycling of intracellular material for biosynthesis, PDAC cells also have the ability to internalize extracellular macromolecules such as proteins and lipids through an endocytic process called macropinocytosis. After being internalized, the macromolecules are carried through large vesicles, the

macropinosomes, which ultimately fuse, as with autophagosomes during autophagy, with lysosomes [44], where the degradation of their components occurs. Degradation products are eventually transported from lysosomes and used to fuel other biosynthetic processes. Several studies have demonstrated that KRAS oncogenic mutations, including KRAS mutations found in human PDAC tumor cells, can strongly upregulate the process of macropinocytosis [45].

Macropinocytosis of serum proteins such as albumin has been shown to be a vital source of amino acid supplementation in PDAC cells undergoing glutamine starvation, and PDAC cell treatment with inhibitors that block macropinocytosis, as for example the endocytosis of albumin, can suppress tumor cell proliferation in vitro and tumor development in vivo [15, 45]. In addition, macropinocytosis can be used to internalize both extracellular material and membrane receptors. Thus, oncogenic KRAS-transformed primary pancreatic ductal cells have been shown to also internalize extracellular lipids to promote their proliferation [46, 47], while other cancer cells have also been shown to internalize extracellular ATP to support ATP-consuming biosynthetic processes [48]. In addition to accumulating soluble nutrients such as proteins and ATP, cancer cells are known to internalize a class of secreted vesicles, called exosomes or microvesicles, through macropinocytosis [49]. For PDAC tumor cells, internalizing exosomes requires KRAS and EGFR-dependent macropinocytosis [49], while other cell types, such as cancer-associated fibroblasts (CAFs), do not apparently require oncogenic KRAS signaling to internalize exosomes [50]. Interestingly, PDAC tumor cells can also release exosomes that may induce dysfunction in normal cells, although the consequences of this for disease progression are currently less clear. Thus, PDAC tumor cells have been shown to release exosomes that can be internalized by normal cells such as pancreatic β-cells, or subcutaneous adipose tissues, to negatively impact insulin secretion or stimulate lipid breakdown, respectively [51].

As cancer cells frequently employ macropinocytosis to aid in receptor regulation and internalize essential metabolites, extensive efforts have been underway in utilizing macropinocytosis therapeutically to deliver cytotoxic drugs specifically into PDAC and other cancers. Some anticancer agents innately undergo macropinocytosis, such as AS1411, which internalizes into various cancer cells through cell-surface nucleolin-dependent mechanisms that activate macropinocytosis only in malignant cells [52]. Other therapeutics specifically target cell surface receptors that may trigger macropinocytosis. For example, therapeutic drugs conjugated with peptides that target a combination of proteoglycans and keratinocyte growth factor receptors (KGFR) can selectively internalize into and kill KGFR-expressing lung cancer cells via macropinocytosis [53].

Another intriguing therapeutic front includes conjugating cytotoxic drugs onto albumin not only to enhance drug pharmacokinetics, but also because albumin has long been observed to accumulate within solid tumors through macropinocytosis [54]. An example includes the FDA-approved nanoparticle albumin-bound form of paclitaxel (nab-paclitaxel or Abraxane®) for treating multiple cancers, including PDAC [1]. However, cancers are still able to overcome these drugs through acquired resistance, likely via differential regulation of proteins that regulate macropinocytosis,

including cytoskeletal and lipid metabolism proteins [55], or through increased expression of drug exporters such as P-glycoprotein [56]. Other albumin-based conjugates targeting folate receptors have also demonstrated efficient delivery of cytotoxic compounds specifically into cancer cells [57]. Finally, as both the autophagic and macropinocytotic pathway converge into lysosomal uptake and digestion of macromolecules, the lysosome can be considered as a therapeutic target for both processes [11].

Redox Balance and Reactive Oxygen Species

Reactive Oxygen species (ROS) are damaging metabolic byproducts generated upon cellular metabolic processes, including oxidative phosphorylation in mitochondria and the action of cytosolic or membrane-associated nicotinamide adenine dinucleotide phosphate (NADPH) oxidases. They are responsible for damage via oxidation of proteins, DNA, and lipids, and when left unmanaged can lead to cell death. Furthermore, the oxidation of DNA is one of the leading causative factors of mutations through generation of 8-hydroxy-2′-deoxyguanosine [58]. However, ROS should no longer considered as simply a damaging byproduct of metabolism as they are now known to also play a significant role in regulating multiple cellular signaling processes, including immune responses, inflammation, adhesion, and cell migration [59].

The generation of ROS has been demonstrated to be crucial for both KRAS transformation and KRAS-driven PDAC tumor expansion [60, 61]. As mentioned earlier, PDAC is characterized by a predominant desmoplastic response, and the activation of PSCs appears to be mainly responsible for the desmoplasia typical of PDAC [14]. ROS may here be an important mediator in the activation of PSCs in this process. During hypoxia, ROS can activate PSCs by stabilization of the transcription factor hypoxia-inducible factor 1α (HIF-1α) and upregulation of the zinc finger transcription factor GLI1 (also known as glioma-associated oncogene) and promote release of other cytokines and growth factors such as IL-6, SDF-1, and VEGF-A to promote pancreatic cancer cell invasion [62]. ROS can also act as an adaptive strategy to inhibit autophagic cell death and its antiautophagic effect may be mediated by upregulating AKT/mTOR signaling in PDAC [63]. In PDAC, the presence of oncogenic KRAS might increase cytoplasmic ROS production through activating NADPH oxidase 4 (Nox4), which is regulated by mitogen-activated protein kinase (MAPK) signaling [64, 65]. As part of the desmoplastic reaction, extracellular components such as fibronectin and laminin may also positively promote Nox4 expression in a 5-lipoxygenase-dependent manner [66]. Oncogenic KRAS is also known to favor the generation of one type of ROS species, superoxide anion, by upregulating the levels of NADPH oxidase 2 (Nox2), an enzyme responsible for electron transfer from NADPH to oxygen molecules. Nox2 activity may critical for PDAC devolvement as Nox2 inhibition in PDAC cell lines can hamper clonal expansion [61]. Relatively similar results have been generated after Nox4 inhibition, indicating that Nox4 (which directly generates an alternative ROS

species, hydrogen peroxide) is also important for PDAC survival [67]. Reduced NADPH enables the preservation of the pool of reduced glutathione, which is essential for subsequent glutathione oxidation, a crucial event for the down-regulation of intracellular ROS levels. The redox capacity of the cells is maintained through the NADP+/NADPH balance that controls recycling of oxidized glutathione [68]. Elevated levels of intracellular ROS within PDAC tumor cells likely promote the progression of pancreatic cancer in the following ways: (1) supporting cell proliferation and survival [64, 66]; (2) promoting angiogenesis via increasing expression of IL-8 [69]; and (3) inducing invasion and metastasis through promoting EMT [70], and increasing the expression of matrix metalloproteases (MMPs) [71].

Cellular control of ROS levels occurs principally through the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) transcription factor that promotes the transcription of various genes essential for ROS detoxification. Such genes include glutathione reductase, NADPH:quinone oxidoreductase 1 (NQO1), thioredoxin, as well as enzymes related to NADPH production such as malic enzyme ME1 [72]. Indeed, Nrf2 has been shown to be overexpressed and activated in PDAC and enables the tumor cells to elicit a sustained ROS detoxification response critical for KRAS-induced tumorigenesis in PDAC models [73]. Therefore, PDAC tumor cells utilize a number of different mechanisms in order to utilize, but carefully control, increased ROS levels to promote tumor survival and proliferation.

Glucose Metabolism

Glucose is the principal growth-supporting substrate in cancer cells and can act as a major provider of carbon for biosynthesis of various macromolecules. Cancer cells are now appreciated to exhibit an aberrant metabolic profile that differs from that of their differentiated counterparts [74]. A major manifestation of this profile is that the presence of oxygen does not restrict glycolysis. This phenomenon, termed aerobic glycolysis and described first by Otto Warburg, is the capacity of cancer cells to metabolize glucose even in the presence of sufficient oxygen, producing lactate [75, 76]. Many human tumors have now been shown to exhibit augmented glucose acquisition coupled to increased flux through downstream glycolytic metabolic pathways. Thus, it is not surprising that mutations in KRAS found in PDAC (as well as other oncogenes and tumor suppressors) reprogram cellular metabolism by acting upon both acquisition and metabolic flux of glucose [60]. Perhaps surprisingly however, in the vascular-poor PDAC microenvironment, overall levels of glucose, and glucose uptake, are thought to be modest compared with other tumor types, and steady-state glucose concentrations have been found not to be significantly elevated compared with normal pancreatic tissue [15]. However despite this, higher levels of glucose uptake (determined by 18F-fluoro-2-deoxyglucose positron emission tomography, FDG-PET) and expression of the glucose transporter GLUT1 have been shown to correlate with poor prognosis in PDAC [77]. Moreover, KRAS-driven alterations in glucose uptake and utilization have been shown to be required,

at least in part, for PDA tumorigenesis [78]. This increased glucose uptake might be facilitated further in PDAC lacking TP53 function, as expression of wild-type TP53 negatively regulates the expression of two different glucose transporters, GLUT1 and GLUT3 [79]. As might be anticipated, in an inducible transgenic GEM model, KRAS silencing strongly reduces glucose uptake and is associated with down-regulation of GLUT1 and multiple enzymes involved in subsequent stages of glycolysis [11, 78]. Indeed, although mutant KRAS can clearly activate the expression of several glycolytic enzymes and alter the glycolytic pathway flux [78], other mechanisms, including hypoxia, have similarly been shown to activate glycolytic enzyme gene expression in PDAC [80]. In contrast, mitochondrial metabolism/ATP generation is likely to be contributed to mainly by glutamine carbon in PDAC cell lines [78, 81].

With more glucose entering PDAC tumor cells, six-carbon units can be diverted into parallel biosynthetic routes, particularly via recruitment of glucose-6-phosphate and other glucose derivatives into both the nonoxidative pentose phosphate pathway (PPP) and hexosamine biosynthesis pathway (HBP) [78]. These twin alterations appear to be KRAS dependent in PDAC and occur via increased expression of two PPP enzymes (ribose-5-phosphate isomerase A and ribulose-5-phosphate-3-epimerase) that promote increased flux of ribulose-5-phosphate (R5P) through the nonoxidative PPP, as well as upregulation of the first enzyme in the HBP pathway, glutamine fructose-6-phosphate amidotransferase (GFPT1) [78]. Additionally however, hypoxia-driven HIF-1α stabilization can also enhance the nonoxidative arm of the PPP by increasing the expression of transketolase genes [82]. This unusual reliance upon the nonoxidative PPP may itself represent a therapeutic target in PDAC, as normal pancreatic cells are thought to generate R5P mainly via the oxidative phase of the PPP [83].

For HBP, the metabolic products are uridine diphosphate-*N*-acetylglucosamine and other nucleotide hexosamines which are major substrates for protein and lipid glycosylation [84]. Indeed, following suppression of KRAS in PDAC, the overall O-glycosylation and tumorigenicity has been found to be reduced dramatically [78]. Excessive O-glycosylation has been previously described in PDAC cells as eliciting an antiapoptotic effect by modulation of nuclear factor-kappa-B (NF-κB) [85]. It should also be noted that in hypoxic conditions, the levels of O-glycosylation in proteins are also thought to be elevated, possibly as an adaptive response to stabilize proteins important for the survival of cells under conditions of low nutrients and oxygen [86]. Thus both KRAS and a hypoxic microenvironment may synergize to elevate O-glycosylation, thereby contributing to PDAC tumor cell survival.

Although such metabolic diversions permit biosynthetic intermediates to be synthesized, the major fate of glucose in PDAC remains lactate [86], converted from pyruvate via lactate dehydrogenase (LDH). PDAC cells are known to alter the flux of this conversion of glucose to lactate in two ways: first, via a KRAS-driven increase in LDHA transcription [78]; and second, by deacetylation of lysine 5 in the LDHA protein, which acts to promote enzymatic activity [87]. To combat the increased accumulation of lactate, PDAC tumor cells also enhance the mechanisms of lactate efflux to the extracellular environment. This occurs in at least three ways.

Firstly, via a combined upregulation of the monocarboxylate transporters for lactate, MCT1 and MCT4, the latter particularly in hypoxic tumor regions [86]. Secondly, by upregulation of a specific G-protein-coupled receptor for lactate, GPR81, which can increase the expression of lactate transporters, and thirdly via increased expression of CD147 that acts as a chaperone for newly synthesized MCT transporters [10, 80].

Given the large production and efflux of lactate, other consequences might be anticipated. Indeed, the use of lactate as an alternative fuel for biosynthesis in some PDAC has been suggested, with lactate produced by PDAC tumor cells in hypoxic areas of the tumor feeding PDAC tumor cells in normoxic areas [86]. Lactate secretion also has been shown to have unexpected effects upon epithelial-stromal interactions. Thus lactate secreted from PDAC cells has been shown to contribute to polarization of a population of immunosuppressive macrophages [88]. Although the full consequences of excess lactate in the PDAC microenvironment remain to be established, in other cancers increased levels of lactate efflux have various other tumor-promoting effects. These include promoting the emergence of an immune-permissive microenvironment by attenuating monocyte migration [89] and dendritic [90] and T cell activation [91]. Furthermore, lactate accumulation is important to promote angiogenesis. Thus, lactate can induce secretion of the proangiogenic factor VEGF from tumor-associated stromal cells [92], while increased levels of lactate can stimulate hyaluronic acid production by fibroblasts, which may contribute to subsequent tumor invasiveness [93]. The final step of glycolysis, the conversion of pyruvate to lactate by LDH, is required to regenerate NAD+ and thus to facilitate continued cycles of glycolysis in PDAC. Thus, LDH represents a potentially attractive drug target in PDAC, as blocking lactate production would be expected to inhibit glycolysis. Indeed, FX11, an inhibitor of LDH [94] has been shown to reduce growth and induce apoptosis in PDAC PDXs in a preclinical study [95].

Glutamine Metabolism

The unexpected finding that the generation of pentoses is uncoupled from NADPH generation in PDAC, with a reliance instead upon the nonoxidative arm of the PPP [81], led to the issue of understanding how PDAC tumor cells can then generate enough NADPH to maintain redox homeostasis. An apparently PDAC-specific glutamine-consuming pathway generating NADPH identified in PDAC [78] has provided a potential explanation for this issue of NADPH deficit. Perhaps unsurprisingly, given its relative abundance in blood plasma [96], the amino acid glutamine plays critical metabolic roles in PDAC, particularly in maintenance of redox homeostasis [11].

In addition to its role in protein biosynthesis, glutamine acts as a major source of carbon and nitrogen for biosynthesis in proliferating cells [96]. PDAC cells grown in culture are known to require glutamine for both proliferation and redox balance [81]. Redox balance is thought to be achieved by two means: by increased generation of the antioxidant glutathione from glutamine-derived glutamate; by utilization of an unusual method of production of NADPH [81], which is itself involved in recycling

of oxidized glutathione and in other reducing reactions. In regard to glutamine-derived glutathione, glutathione abundance has been found to be increased in PDAC in comparison to normal pancreatic tissue, with inhibition of glutathione synthesis in vitro inducing growth inhibition and promoting apoptosis [97]. This latter pathway appears to be driven by oncogenic KRAS and converts glutamine-derived carbon into aspartate. This occurs within mitochondria via a series of reactions that firstly utilize the mitochondrial Asp aminotransferase (GOT2) [81]. Glutamine-derived aspartate is then transported into the cytosol and acted upon by a second enzyme, aspartate aminotransferase (GOT1), generating oxaloacetic acid (OAA). OAA is then converted to pyruvate by the cytoplasmic form of malic enzyme 1 (ME1), yielding NADPH [81]. This pathway of glutamine metabolism may also represent a specific metabolic vulnerability in PDAC as is has not been found to be used in normal pancreatic cells [81].

In addition to activation of the above mitochondrial/cytosolic pathway, as discussed previously mutant KRAS also initiates a nuclear Nrf2 transcription factor-dependent ROS detoxification program [72, 98]. The Nrf2 transcriptional response is normally activated in most cells by redox stress [99]; however, mutant KRAS constitutively activates this transcriptional program to suppress ROS and promote PDAC tumorigenesis and proliferation [100]. The Nrf2-directed transcriptional response has also been shown to redirect glucose and glutamine into anabolic and antioxidant pathways [72, 98]. Nrf2 also increases ME1 expression, thereby linking mutant KRAS with increased flux through ME1, generating increased NADPH to assist redox homeostasis [81]. Interestingly, the expression of malic enzymes ME1 and ME2 are transcriptionally repressed by wild-type TP53 [79], indicating again that the loss of TP53 function in advanced PDAC might synergize with KRAS to further increase metabolic flux to generate increased NADPH levels. Underscoring the importance of this antioxidant pathway, inhibition of these enzymes involved in NADPH generation in PDAC impairs viability both in vitro and in vivo [81]. Since Nrf2 also activates glutathione biosynthesis [98], mutant KRAS appears to enhance antioxidant defense in PDAC both via enhanced NADPH-dependent recycling of oxidized glutathione, and by an Nrf-2-dependent increase in glutathione synthesis.

In addition to contributing to redox homeostasis, glutamine plays a key role in providing substrates for biosynthesis via glutaminolysis by generating α-ketoglutarate (α-KG). α-KG is produced via the action of glutamate dehydrogenase (GLUD1) upon glutamate, with glutamate produced via breakdown of glutamine by glutaminases (GLSs) in mitochondria. Ultimately, this leads to the generation of α-KG–derived intermediates from the TCA cycle that are subsequently utilized in fatty acid (FA) synthesis [96], or in generating nonessential amino acids with glutamine acting as the nitrogen donor [96]. In PDAC tumor cells, KRAS acts to increase GOT1 while suppressing GLUD1 expression [81], suggesting that PDAC tumor cells preferentially utilize glutamine to counteract redox homeostasis and promote ROS detoxification, rather than to promote biosynthesis. Since ROS, generated by the action of NADPH oxidases Nox2 and Nox4, is increased by KRAS and is itself required for clonogenic growth [61, 67] and EMT [101] in

PDAC, it appears that the benefits of ROS for PDAC tumor cells are counteracted by glutamine-derived NADPH, thereby preventing excessive ROS inducing deleterious effects upon viability.

Alterations in Lipid Metabolism in PDAC

Metabolomic studies in the lipid metabolism mechanisms of PDAC cancers have surprisingly shown that they bear a lower fatty acid (FA) content when compared to normal surrounding tissue [43, 102]. However, when a study assessed the effects of dietary fat on a GEMM model of PDAC development, it was demonstrated that the high levels of lipids obtained from the diet led to a KRAS-COX2-dependent increase in the formation of PanINs and PDACs [103]. Indeed, high-fat diets and obesity are strongly linked with PDAC incidence [104], suggestive of a role of lipids in PDAC initiation or progression. Interestingly, PDAC tumor lines cultured with oleic (a monounsaturated omega-9 fatty acid) or linoleic acid (a polyunsaturated omega-6 fatty acid) have increased the rates of proliferation [105], suggesting that some FAs may be limiting for tumor growth, and rapidly metabolized. Consistent with this notion, KRAS transformation of normal immortalized pancreatic ductal epithelial cells (HPNE) is also known to increase scavenging of extracellular lysophospholipids as an alternative source of FAs [46], while PDAC cells exhibit increased acquisition of cholesterol [47]. Fatty acid synthase has also been reported to be upregulated in PDAC, likely downstream of KRAS via MAPK signaling, with increased expression correlating with poor prognosis [106].

Metabolic Crosstalk in the PDAC Tumor Microenvironment

One of the most prominent characteristics of PDA is an intense desmoplastic reaction around the tumor. Surrounding stroma occupies the largest volume of the tumor and it is developed from noncancerous cells including pancreatic stellate cells, immune cells, and endothelial cells surrounded by a dense extracellular matrix rich in collagen and hyaluronic acid [107]. As previously discussed, the formation of this unusual stroma induces a poorly vascularised microenvironment that limits the diffusion of oxygen and nutrients alike in PDAC [15, 108]. As a result of the limited vasculature, carcinomas are hypoxic and require adaptation to sustain their growth [11]. PSCs are the most abundant resident fibroblast-like cells present in lesions of the pancreas and a variety of evidence indicates are responsible for the desmoplastic reaction [14]. In healthy exocrine pancreas, PSCs are thought to maintain normal tissue architecture via regulation of the synthesis and degradation of extracellular matrix (ECM) proteins [109]. Following injury or inflammation, PSCs transform from their quiescent phase into an activated, myofibroblast-like phenotype, secreting excessive amounts of ECM proteins leading to the fibrosis typical of chronic pancreatitis and PDAC [109]. Furthermore, PSCs can also regulate the turnover of the tumor stroma through the expression of matrix metalloproteinases

such as MMP1 and MMP2 [110]. Targeting the stroma through enzymatic modulation of hyaluronic acid enhances the delivery of chemotherapeutic agents and therefore increases the cytotoxic potential of those drugs [13].

PDAC cells can initiate a reciprocal signaling network between the tumor cells and the neighboring PSCs [111]. In the face of poor nutrient supply from the vasculature, metabolites are accessed by PDAC tumor cells from surrounding stromal cells [112]. PSCs have for example been found to release nonessential amino acids (NEAAs) in response to culture with PDAC tumor cells [112]. PDAC cells in particular consume PSC-derived alanine and use it to fuel additional metabolic processes including mitochondrial metabolism, fatty acid synthesis, and synthesis of other amino acids [112]. The mechanism involved in such nutrient accessing by PDAC tumor cells appears to be via autophagy that is induced within PSCs. Indeed, the blockage of autophagy prevents alanine release from PSCs, although the specific mechanism of alanine release remains to be established [112].

Another component of the tumor microenvironment is infiltrating leucocytes. The T-lymphocytes account for the most abundant cell type in adaptive immunity and are responsible for the identification of foreign antigens which have been previously processed and presented by antigen-presenting cells (APCs), such as dendritic cells and macrophages. Antigen recognition mediated through T-cell-APC interaction leads to T-cell activation, clonal expansion, and migration toward the antigens where the T-cells exert various effects according to their subtype.

The T-cells are divided into CD4+ T-helper cells which are further subdivided into Th1, Th2, and Th17 populations; CD8+ cytotoxic cells; and regulatory T-cells (Tregs). Each of the T-cell subtypes has distinct functions; the polarization of the CD4+ cell populations is triggered by different cytokine combinations, and therefore Th1, Th2, and Th17 cells display differential cytokine production patterns with opposing effects. The CD8+ cells are responsible for cell-mediated cytotoxicity whereas the Tregs exhibit immunosuppressive properties [115].

CD8+ cells are capable of infiltrating tumor sites and killing cancer cells and have therefore been associated with better prognosis in numerous cancer types, among them melanoma, head and neck cancer, lung, breast, and colon cancer. However, human PDAC is characterized by poor CD8+ T-cell infiltration to the tumor site. This could be attributed to the low mutation rate observed in PDAC in contrast to other KRAS-induced cancers (e.g., lung cancer), which leads to the limited formation of neoantigens that could be recognized by the T-cells [113]. Furthermore, the formation of a tumor microenvironment with a predominant presence of Th2 cells, Tregs, MDSCs, and immunosuppressive cytokines further impedes the CD8+ T-cell activation, favoring tumor sustainability and progression.

CD4+ T-cells play a pivotal role in the regulation of CD8+ T-cell function and therefore the predominance of the Th2 cells in the PDAC tumor milieu are thought to significantly hamper the activation of the CD8+ T-cells through the production of immunosuppressive cytokines such as IL-4, IL-5, and IL-10 [114]. On the other hand, the Th1 cells can suppress tumor proliferation by producing IL-2 and IFN-g which have a proinflammatory effects and are essential for CD8+ T-cell activation and proliferation [115]. Therefore, the Th1/Th2 ratio is crucial in order to determine

whether the elicited immune response at the tumor site will either suppress or promote tumour growth [115].

Aside from the large amounts of Th2 cells, the PDAC tumor microenvironment is also preoccupied with another Tregs, which have a prominent immunosuppressive role [116]. The Tregs can inhibit the function of several immune cell types such as natural killer cells (NK), B-cells, and dendritic cells through granzyme B production but can also secrete several cytokines such as TGF-β1, IL-6, TNFa, and the receptor activator of NF-κB, which strongly enhance further tumor development [117].

The metabolic landscape of pancreatic cancer microenvironment further enhances tumor progression by dampening the immune response. Infiltration of immune cells can be observed early in the development of pancreatic cancer. PanINs initiate the accumulation of leukocytes that consist mostly of macrophages, $CD4^+$ T regulatory cells (Tregs), and myeloid-derived suppressor cells (MDSC) that all act in an immunosuppressive manner [118]. Effector $CD8^+$ T cells that could suppress tumor growth have only been found in low numbers and with no indication of activity [118].

One of the underlying causes for the lack of significant effector immunity could be the nutrient competition between immune cells and cancer cells. T cells are also known to utilize aerobic glycolysis to support their high needs of energy upon clonal expansion and secretion of various cytokines [119]. Glucose deprivation of T cells leads to "exhaustion," a phenomenon that blocks effector function of T cells and leads to low levels of IFN-γ production [120]. Such an effect has been observed in the studies of sarcoma and melanoma models where the existing pool of glucose in the tumor microenvironment is diminished due to excessive glycolytic metabolism of cancer cells, leading to glucose restriction of nonregulatory CD4+ T helper cells (TH1) [120]. TH1 cells subsequently fail to mount a prominent presentation of the antigen or recruit effector cells.

Conclusion

Altered metabolism is now recognized as an important hallmark of cancer [121], and efforts are ongoing to therapeutically exploit some of these metabolic differences between normal and cancer cells [12]. In the case of pancreatic cancer, metabolism is known now to be significantly impacted upon by mutation of the KRAS proto-oncogene, presenting exciting potential opportunities for selective targeting of metabolism in PDAC. Together with KRAS mutations, the hypoxic microenvironment generated by stromal deposition similarly forces PDAC tumor cells to rely on alternative sources of nutrients, and to utilize unique methods to obtain them. Unfortunately however, there are currently no clinical useful mutant KRAS inhibitors, and only a few clinically viable Ras-effector treatments that might be the most direct way to combat altered metabolism in PDAC deriving from mutant KRAS.

Of other mechanisms that might be therapeutically viable in PDAC, recent work indicates that constitutive autophagy – a major feature of PDAC tumor cells – and glutamine utilization, fuelling NADPH reserve to maintain redox homeostasis,

would appear to be two of the most promising avenues for therapeutic intervention. Inhibitors of autophagy, such as HCQ, are currently being assessed in PDAC clinical trials, although HCQ pharmacostability is likely to be an issue, as is penetrance of any small molecule inhibitor through the dense stroma and hypoxic PDAC microenvironment. In the case of therapeutic targeting glutamine utilization, the use of an apparently PDAC-specific breakdown of glutamine is particularly attractive, although whether there are other normal tissues that might use such a strategy would need to be more completely explored.

Confounding such approaches however will likely be the presence within PDAC of distinct metabolic subtypes and significant intratumoral heterogeneity. These might allow significant tumor evolution during the course of any therapy aimed at targeting one specific metabolic feature [122]. In addition, unlike specific fixed genetic alterations such as mutant KRAS, metabolic networks are now known to exhibit inherent plasticity and are therefore likely to be rewired in the face of targeted therapies unless cell death is rapidly obtained [123]. Finally, although there is great interest in dissecting immune responses in the PDAC microenvironment and evolving immunotherapies that attack PDAC cancer cells [124], glycolysis inhibitors targeting PDAC tumor cells may further interfere, in unknown ways, with antitumor immune responses by blocking metabolic alterations also critical for immune cell activation.

Cross-References

- ▶ Approaching Pancreatic Cancer Phenotypes via Metabolomics
- ▶ Emerging Therapeutic Targets in Pancreatic Adenocarcinoma
- ▶ Role of Tumor-Stromal Interactions in Pancreatic Cancer Invasion and Metastases
- ▶ Stromal Inflammation in Pancreatic Cancer: Mechanisms and Translational Applications
- ▶ Vaccine Therapy and Immunotherapy for Pancreatic Cancer

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Secondary Screening for Inherited Pancreatic Ductal Adenocarcinoma

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Abstract

The prevalence of pancreatic cancer is too low, and the accuracy of current screening methods is not high enough to permit general population screening. Secondary screening in high-risk groups may be possible for the disease or its precursors. Pilot screening studies have been initiated and are generating data on the nature of inherited predisposition and the early stages of cancer development. It is already apparent that the specificity and sensitivity of secondary screening tests need to be improved. In this chapter, the preliminary evidence from the pioneering screening studies will be considered in order to discuss which participants should be recruited into future pilot studies and how biomarkers may in future be combined with imaging to reduce the number of missed cancers and premature surgical interventions.

Keywords

Pancreatic cancer · Inherited diseases · Risk · Screening

Introduction

Pancreatic ductal adenocarcinoma (PDAC) remains one of the most lethal cancers, with a 5-year survival of about 6% [1]. In the USA, it is estimated that in 2016, there are roughly 53,070 cases of pancreatic cancer diagnosed with 41,780 deaths [1]. The incidence of PDAC has been rising, and it is predicted to be the second leading cause of cancer deaths by 2030 [2]. Despite significant improvements in cancer treatment, prognosis remains poor as the majority of patients with PDAC (80–90%) [3, 4] have unresectable disease at diagnosis, due to late presentation.

Surgery remains the only potentially curative treatment, but even after resection, 5-year survival rates are less than 10% [5]. Adjuvant chemotherapy with gemcitabine and capecitabine following resection has recently been shown to convey a significant improvement in 5-year survival 29% over the previous standard treatment with gemcitabine monotherapy of 16% [6].

The cohort series of partial pancreato-duodenectomy (with variable neoadjuvant and adjuvant therapies) from Johns Hopkins Medical, showed that the median survival rate for cancers <3 cm was 21 months, and the 1-, 2-, and 5-year survival

rates were 73%, 45%, and 23%, respectively [7]. Smaller pancreas cancers without perineural or lympho-vascular invasion had a better prognosis. For cancers >3 cm, the median survival was 15 months, and the 1-, 2-, and 5-year survival rates were 59%, 31%, and 4%, respectively. PDAC with the best chance of a cure are small <1 cm, well-differentiated stage 1 cancers, with postresection 5-year survival rate of up to 75%. To improve survival early detection is necessary but small pancreatic cancers are generally asymptomatic and so patients with such tumors do not present for clinical investigation. Imaging techniques are sensitive, but do not approach the level of 100% specificity, which is required to avoid an excess number of false positives given the relatively low prevalence of the disease [8].

Pancreatic cancer represents only 3% of estimated new cancers each year [9], but is the fourth most common cause of cancer mortality. By 2030, it is estimated to be the second leading cause of cancer death [2]. Approximately, 5–10% of PDAC cases have an inherited genetic component. With an ever aging population, the incidence and frequency of PDAC will increase. There is a need for focused affordable screening in high-risk populations based on reliable biomarkers and efficient imaging modalities. The nature of genetic susceptibility was discussed in a previous chapter of this handbook. In this chapter, the discussion will be expanded to address the question of how great the risk needs to be in order to justify secondary screening.

Who to Screen

Primary Versus Secondary Screening

To be effective any test should provide a True Positive:False Positive ratio of >1.0. The True Positive:False Positive ratio is affected by the sensitivity and specificity of the test and the prevalence of the disease in the population being screened. The age adjusted incidence of pancreatic cancer is up to 10 per 100^5 overall and even for those over 75 years of age (the highest risk age group), it is less than 120 per 100^5 [10]. This means that in a screened population of 10^3 participants it would be reasonable to expect just one true positive individual. Unless the screening test was better than 99.9% specific there would be many more false positives than true positives. For example, assuming a test with sensitivity and specificity both of 85% and a partially enriched screening population with a prevalence of 20 per 100^5, screening 10^5 individuals would produce 17 True Positives, 84,983 False Negatives, 14,997 False Positives, and three False Negatives with a True Positive: False Positive ratio of 0.001. So, primary screening of the general population is not an option. The positive predictive value of a test can be improved by selecting a population with an increased prevalence of the disease being tested for, such as age, smoking history and a potential or actual genetic predisposition to pancreatic cancer. Secondary screening requires a highly enriched population with as high a prevalence as possible and a highly sensitive test. As any potentially curative treatment will require surgical resection of the pancreas, which has considerable morbidity and a small mortality rate [11], the specificity must approach 100%.

Screening Consensus

Consensus recommendations for secondary screening of high-risk groups were initially proposed at the Fourth International Symposium on Inherited Diseases of the Pancreas in 2003 [12]. It was concluded that secondary screening should only be carried out in patients with a strong genetic susceptibility. Hereditary pancreatitis (HP, with PRSS1 mutations) was included as was Peutz-Jeghers' syndrome (PJS, with STK11/LKB1 mutations). The International Cancer of the Pancreas Screening Consortium (CAPS) was formed in 2010 with the specific objective of developing statements on screening in individuals at risk (IAR) with an inherited disposition to PDAC. From the 2011 CAPS meeting, the group recommended screening should be undertaken in IARs to detect relevant lesions, in participants who would be suitable for potential surgical resection. Ideally, this should be at a tertiary center as part of a recognized research program [13]. IARs included familial pancreatic cancer (FPC), PJS, familial atypical multiple mole melanoma (FAMMM) syndrome with *CDKN2A* mutations, breast-ovarian syndrome with *BRCA2* mutations, and hereditary non-polyposis colorectal cancer (HNPCC) mutation carriers with >1 affected first-degree relatives (FDR). No consensus was reached on the ages for initiating and stopping screening, on the imaging modality of choice (although endoscopic ultrasound (EUS) and MRI were recommended), or on screening intervals. Defining a strong genetic predisposition is not straightforward and involves a complex assessment of polygenic and environmental factors. In the end, the experts at the CAPS meeting reached a compromise on a select few conditions and what constituted an individual at risk.

Individuals at Risk (IAR)

There are numerous factors that increase the risk of developing PDAC, including age, tobacco consumption, non-O blood groups, chronic pancreatitis, late-onset diabetes mellitus, hereditary pancreatitis, cystic fibrosis, certain cancer family syndromes, and a family history of PDAC. The lifetime risk of PDAC in the general population is around 1%. Individuals with an at least 5–10-fold increased risk for PDAC are deemed IAR and are considered to be good candidates for screening.

An FPC family is defined as having at least two FDRs, or three or more second-degree relatives, with pancreatic cancer. Despite pancreatic cancer being relatively uncommon, clusters of cases will occur by chance. If the cluster is random then prospective incidence of pancreatic cancer would be equivalent to the general population. A family history will make it more likely that an individual is carrying some high-risk allele of a gene, but the majority of people with a family history will not have any particular genetic predisposition and instead family members may share some environmental risk factor. Stratification of risk is based on a family history, the greater the chance that a family has autosomal dominant genetic predisposition, the greater the risk estimate. Klein et al. have shown that the risk of PDAC in an individual with two FDRs affected is 6.4-fold greater than someone with no

affected FDRs (8–12% lifetime risk), whereas an individual with three FDRs can be estimated to have a 32-fold increased PDAC risk (40% lifetime risk) [14].

Risk can be further refined if an individual has undergone specific genetic testing to identify mutations in known pancreatic cancer susceptibility genes. Several germline gene mutations have been identified as increasing PDAC risk [12]. The relative risk of PDAC associated with each gene mutation is summarized in Table 1.

Hereditary pancreatitis (HP) is characterized by recurrent attacks of acute pancreatitis, progressing to chronic pancreatitis with a family history of pancreatitis consistent with an autosomal dominant inheritance pattern and/or the presence of a proven known genetic mutation [15]. HP confers an approximate 25–40% lifetime risk of PDAC to the age of 70 years [15]. Sporadic chronic pancreatitis also increases the risk of pancreatic cancer fivefold [16], but the risk is too low to enable effective secondary screening in this group of patients.

Peutz-Jeghers' syndrome (PJS) is an autosomal dominant disorder with increased risk of multiple cancers. The phenotype comprises hamartomatous gastrointestinal polyps, and oro-buccal mucosal pigmented macules most of whom have an STK11 gene mutation with a very high pancreatic with cancer risk [17]. A meta-analysis of 210 patients found the relative risk (95% confidence interval, CI) for all cancers was 15.2 (2, 19) % with an average age of onset of malignancy of 41 years, compared with over 60 years for the general population [18]. The cumulative risk for all cancer was 93% from age 15 to 64 years old. A statistically significant increased relative risk (95% CI) was found for esophagus (57; 2.5, 557%), stomach (213; 96, 368%), small intestine (520; 220, 1306%), colon (84; 47, 137%), pancreas (132; 44, 261%), lung (17.0;5.4, 39%), breast (15.2;7.6, 27%), uterus (16.0; 1.9, 56%), ovary (27; 7.3, 68%), but not testicular or cervical malignancies.

BRCA2 mutation carriers constitute the largest group of mutation carriers at risk for PDAC [19]. The PDAC risk in this group is between 3.5% and 10%, depending on number of affected family members [20]. The PDAC risk would appear to be context specific as not all carriers in a BRCA2 family will develop PDAC. Pandharipande et al. used MRI based simulation screening model for PDAC in BRCA2 families and demonstrated a small life expectancy gain with screening, which was eliminated with a slight increase in surgical mortality rate (>2.3%) [21]. The recommendation was to restrict screening to BRCA2 mutation carriers with at least two FDRs with PDAC.

Familial atypical multiple mole and melanoma (FAMMM) is an autosomal dominant syndrome with a subset of patients with this syndrome harboring mutations in *CDKN2A* (the gene encoding p16 protein), which are frequently found in sporadic pancreatic cancer [22]. The estimated cumulative risk of developing PDAC in CDKN2A is 17% [23].The association between inherited gene mutations and pancreatic cancer in the other syndromes mentioned in Table 1 is small including the risk in hereditary breast-ovarian cancer caused by *BRCA1* mutations [24].

Whole genome sequencing has defined considerable genetic heterogeneity of FPC [25]. Although familial aggregation of pancreatic cancer has been established, the cause of this aggregation in most families is unknown. Roberts et al. sequenced

Table 1 Inherited gene mutations associated with PDAC

Hereditary tumor predisposition syndromes	Gene mutations	% of presumed FPC families	Relative risk	Risk at 70 years of age (%)
None	**None**	–	1	0.5
Peutz–Jeghers syndrome	***STK11/LKB1***	<1	132	30–60
Hereditary pancreatitis	***PRSS1***	0	50–80	25–40
FAMMM	***p16/CDKN2A***	1	20–34	10–17
Hereditary nonpolyposis colon cancer (HNPCC)	**MSH2, MLH1, MSH6, etc.**	<1	8	3.7
Hereditary breast-ovarian cancer	***BRCA1***	<1	Unknown	Unknown
Hereditary breast-ovarian cancer	***BRCA2***	5	3.5–10	3.5
Li–Fraumeni syndrome	**TP53**	<1	<5	<5
Familial adenomatous polyposis	**APC**	<1	<5	<5
Cystic fibrosis	**CFTR**	0	<5	<5
Ataxia telangiectasia	***ATM***	<1	Unknown	Unknown
Possible FPC	***PALB2***	<1	Unknown	Unknown

the germline genomes of 638 patients with FPC and the tumor exomes of 39 familial pancreatic adenocarcinomas. Previously identified FPC susceptibility genes such as BRCA2, CDKN2A, and ATM, were confirmed but novel candidate genes harboring rare, deleterious germline variants were also identified requiring further characterization. The genetic underpinning of inherited pancreatic cancer is highly heterogeneous which has significant implications for the management of patients with FPC. In particular, for screening the implication is that the focus must be heavily reliant on the phenotype of family clusters.

Quantified Risk of Incident Pancreatic Cancer Among First-Degree Relatives of Patients with Familial Pancreatic Cancer

The Johns Hopkins group prospectively quantified the risk of PDAC among first-degree relatives of incident cases in both sporadic forms and those with FPC, defined as kindreds with at least two first-degree relatives with PDAC in families enrolled in the National Familial Pancreas Tumor Registry (NFPTR) [26]. There were 191 families with sporadic pancreatic cancer (without a pair of affected first-degree relatives) and 150 families with FPC including 52 kindreds containing three or more affected members at the time of enrolment. Risk was estimated by comparing observed new cases of PDAC during the observation period with expected numbers based on data in the USA population-based Surveillance, Epidemiology, and End Results (SEER) program. Incidence was estimated using person-years risk analyses. During the

observational period, six incident cases developed in the first-degree relatives, two in the sporadic families, and four in the FPC kindreds.

The PDAC risk in the sporadic kindreds was not significantly greater than expected [observed: expected (O:E) = 6.5 (95% CI = 0.78–23.3)] with an incidence rate of 24.5 per 10^5 per year. The expected incidence of PDAC from the SEER data was 8.8 per 10^5 per year. There was a significant increased risk in first-degree relatives in FPC kindreds (O:E = 18.3; 4.74–44.5), with an incidence of 76.0 per 10^5 per year. In the subset of FPC kindreds with three or more affected family members there was a 57 (12.4–175)-fold increased risk with an incidence of 301.4 per 10^5 per year. In the FPC kindreds, the increased risk appeared to be largely confined to relatives 60 years of age and older.

In an Italian study of 570 index cases of pancreatic cancer, there were 54 who reported of a family history of pancreatic cancer [27]. Pancreatic cancer was significantly increased in first-degree relatives (relative risk at age 85 years = 2.7). Nearly all the risks were explained by just seven families with little increase in remaining 47 families.

These data support the notion of secondary screening, but would not be appropriate for most families with a family history. Identification of the most efficient screening systems is still in development [28].

The more cases there are in a family the more likely it is to be consistent with FPC, but this is balanced by a reduced probability that the family is FPC if there are nonpenetrant members of the kindred.

It is difficult to define nonpenetrant, partly because most FPC kindreds have no identified disease mutation. This problem can be partially resolved by using obligate carriers, for example anyone who has both antecedents and descendants with pancreatic cancer, but at what age can they be described as having past the point at which they should have developed cancer. There is some evidence for anticipation in FPC, with progressively earlier age of cancer with successive generations [29]. Therefore, if an obligate carrier exceeds the age of an affected parent, or exceeds the age of an affected child by at least 10 years, then they can reasonably be defined as nonpenetrant. Figure 1 shows four hypothetical families, each with two members who are affected with pancreatic cancer. Families A and D have the least nonpenetrants and so are the most likely to be FPC kindreds. The seven out of 570 Italian families described by Del Chiaro et al. as being high risk had just one nonpenetrant individual between them [27], limiting screening to apparently fully penetrant families in the Del Chiaro series would therefore have risked missing one genuine family, but would have meant that the majority of false families would not be screened. The probands from families A and D but not B or C would be recruited for screening. Further refinement of selection is possible, for example, the single family with a nonpenetrant had three affected individuals, a criteria of three or more affected per nonpenetrant would mean that none of the high-risk families in the Del Chiaro study would be missed, but a large proportion of false families would be eliminated.

The risk of cancer is not solely determined by the nature of the family. The probability an individual within that family is a carrier must always be taken into

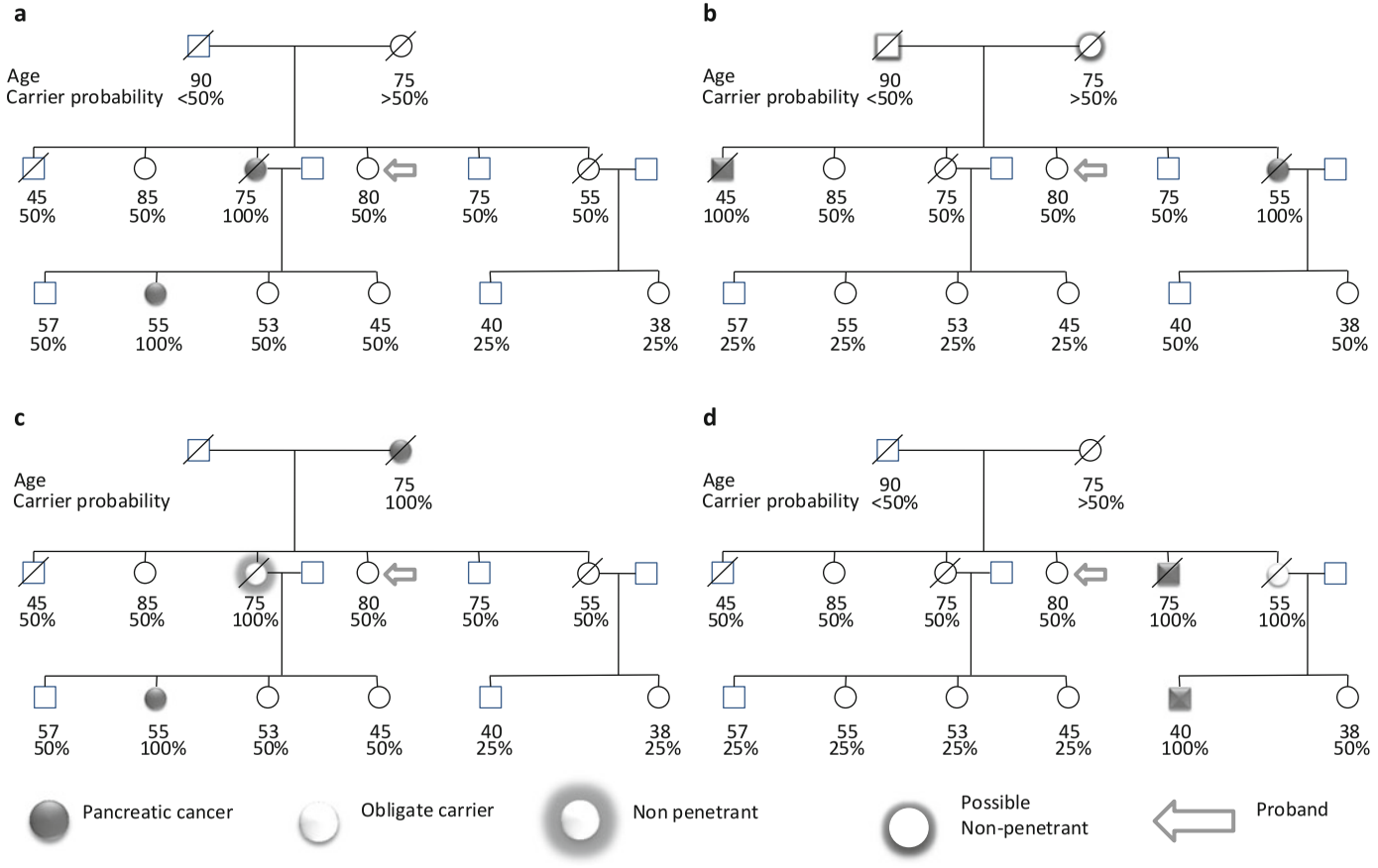

Fig. 1 (continued)

account, the 57-year-old nephew of the proband in family A from Fig. 1 is therefore at more risk than the equivalent nephew in family D. In addition, the age of the individual must be considered.

When to Screen and Risk Stratification

Age Dependent Risk

Age is the greatest risk factor for sporadic pancreatic cancer, below the age of 60 years age adjusted incidence of pancreatic cancer is less than 1 in 10^5, between 60 and 75 years it is close to 40 in 10^5, and over 75 years the incidence approaches 100 per 10^5, [10]. Genetic predisposition could increase cancer risk at specific ages. For example, an affect linked to puberty could increase risk a fixed number of years afterwards, cancer risk before or after this point would be unchanged but overall risk would increase. Alternatively, a risk later in life might be increased but risk at younger ages could be unchanged, thus only late-onset disease would be increased. Comparison of data for pancreatic cancer incidence from the SEER program of the National Cancer Institute in the USA with data for FPC kindreds from the European Registry of Hereditary Pancreatitis and Familial Pancreatic Cancer (EUROPAC) suggests that high risk in FPC can best be modeled by a 120- fold constant factorial increase in risk at each age, rather than a greatly increased risk at particular ages and smaller increases at others [28].

The figure of a 120-fold increase represents an average, some families will have greater factorial increases and some less; equally some individuals will have a greater increased age dependent risk than other at risk members of the family. This is evident from anticipation, which can only be explained if parents have a lower age specific risk than their offspring [29]. Other variables that may affect age specific risk are gender, smoking, and secondary genetic factors.

Men have a slightly increased risk of pancreatic cancer compared to women in the general population [10], but gender makes no significant difference to age of cancer in FPC [29]. Similarly in HP cancer risk does not seem to be significantly affected by

Fig. 1 Consistency with autosomal dominance. All family trees have two affected members with pancreatic cancer, but only family (**a**) and (**d**) have no requirement for anyone to be defined as nonpenetrant. Thus, (**a**) and (**d**) are more likely to be carrying a monogenic predisposition for pancreatic cancer. In family (**a**), one of the proband's parents must be a carrier if the family has FPC, the mother was just 75 (the same age as the proband's affected sister), which means that this parent could have been a carrier who never reached the age of penetrance. In contrast, both parents of the proband in family (**b**) are more than 10 years older than the oldest affected individual. One of the parents must therefore be nonpenetrant. In family (**c**), the 75-year-old sister of the proband must either be nonpentrant or the family does not suffer from FPC. In family (**d**), the 55-year-old sister of the proband must be a carrier if this family does genuinely suffer from FPC, but this individual died 20 years before her affected sister, so again this carrier could have not reached the age of penetrance. Family (**d**) is more likely to be FPC than (**b**) or (**c**), but less likely than family (**a**)

gender [15]. Tobacco smoking is the most important environmental risk factor associated with PDAC and increases the risk of sporadic pancreatic cancer by twofold compared to nonsmokers [30], and accounts for 25% of all PDACs [31], with a strong dose-response relationship [32], so some smokers may be at even greater risk. It seems that smoking is synergistic with family history in predisposing to cancer [33]. In the EUROPAC cohort of FPC families, it is less clear that smoking has a significant impact [29], but there are reports of earlier age of pancreatic cancer even in the context of autosomal dominant predisposition [34]. In HP, smoking is a clear risk factor for pancreatic cancer [35]. Polymorphisms in cell cycle genes are associated with earlier age of onset in sporadic pancreatic cancer [36]. It is possible that these and other polymorphisms will also influence age of onset in FPC.

The age at which to start and stop screening for PDAC is uncertain, and more evidence is required. There was disagreement on this topic at the CAPS summit, where the slight majority of experts (51%) voted to initiate screening at 50 years of age; however, most of the active research programs, and thus support data, start screening around the age of 40–45 years, or at 10 years younger than the youngest case of PDAC diagnosis in the family.

Risk Stratification

The relative lack of success of pilot screening programs for pancreatic cancer can be explained by inclusion of too many low-risk individuals in the screening cohorts. Risk stratification models could be used to optimize screening and surveillance programs, to increase the likelihood of detecting a PDAC and mitigating the unavoidable risks associated with false positive screening results. Risk-prediction models such as Panc-PRO, a Mendelian risk prediction tool for pancreatic cancer and have been developed to help identify individuals in families with HP and FPC who have the highest risk of developing PDAC [37]. Work continues to model risk and it is hoped that these mathematical models will be able to provide age specific risk stratification. They may also have a role in reassuring members of the public who have no elevation in PDAC risk. This would be particularly useful in FPC in the absence of a genetic test. Meanwhile, simple stratification is relied upon, such as above or below 40 years, or a certain number of years before the age of cancer diagnosis in other members of the family.

Alarm Symptoms and Screening for Pancreatic Cancer

Risk stratification could be combined with symptoms [38]. Early alarm symptoms such as dyspepsia, vague upper abdominal pain, anorexia, weight loss, and late-onset diabetes mellitus are easily dismissed in the general population as too unspecific to trigger investigations [39], but as part of a primary screen they could be used to trigger secondary screening. Evidence that diabetes mellitus is a very early symptom of pancreatic cancer is provided by the observation that pancreatic cancer patients

were more likely than controls to have developed hyperglycemia within a 4 year period [40] and such recent onset diabetes often resolved following pancreatic cancer resection indicating that it was the cancer causing the diabetes rather than the diabetes predisposing to cancer [40, 41].

Risk Assessment for Screening

Risk assessment to estimate whether benefits of the entire screening process outweighs any drawbacks should be made before first approaching potential participants. Risks to the participant start with raised anxiety and extend through to a chance of unnecessary pancreatic resection. The principal benefits include reassurance and possible lifesaving early treatment. Unfortunately, in the case of pancreatic cancer there are great gaps in our knowledge which makes risk assessment more an art than a science.

Any risk assessment must start with an estimation of cancer incidence and the maximum number of people screening could possibly benefit. Age adjusted incidence of pancreatic cancer was approximately 10 in 10^5 [10] with 53,070 cases in 2016 [1]. As shown in Fig. 2 a primary screening program with 95% test sensitivity and 99% test specificity aimed at the entire USA population (320 million) would give a gross excess of false positives over true positives. Approximately 10% of pancreatic cancer cases [34] and 1% of the general population [42] report a family history of pancreatic cancer. This shows that for the screening test to have a True Positive:False Positive ratio >1 then a family history with an autosomal dominant pattern of inheritance is required. In the absence of a known mutation, this would need to be at least three first-degree relatives in two or more generations (Fig. 3).

Increasing the stringency of the definition of a family history will further increase the incident population. From the Del Chiaro study of Italian index cases, approximately seven of 54 (13%) of pancreatic cancer patients who report a family history have at least two other cases in the family [27]. By definition, the risk of pancreatic cancer for any individual in a family without any predisposition is independent of family history. Based on the probability of 1% of control families having a single family member with pancreatic cancer [42], the chance of any family having two cases is 0.01%. EUROPAC recruits participants on the familial pancreatic cancer register as families with two or more affected individuals not fulfilling criteria for any other familial cancer syndrome. [29]. In October 2016, 746 of 980 families (76%) were consistent with autosomal dominance. It is unclear how much of this is due to random clustering and how much is due to genuine predisposition. Only 69% of families with just two affected members are consistent with autosomal dominance compared to 88% for families with more than two members. It can therefore be conservatively assumed that 88% of genuine families and 69% of random clusters will be consistent with autosomal dominance; screening 9,288 individuals would give just 90 false positives and still give 273 true positives.

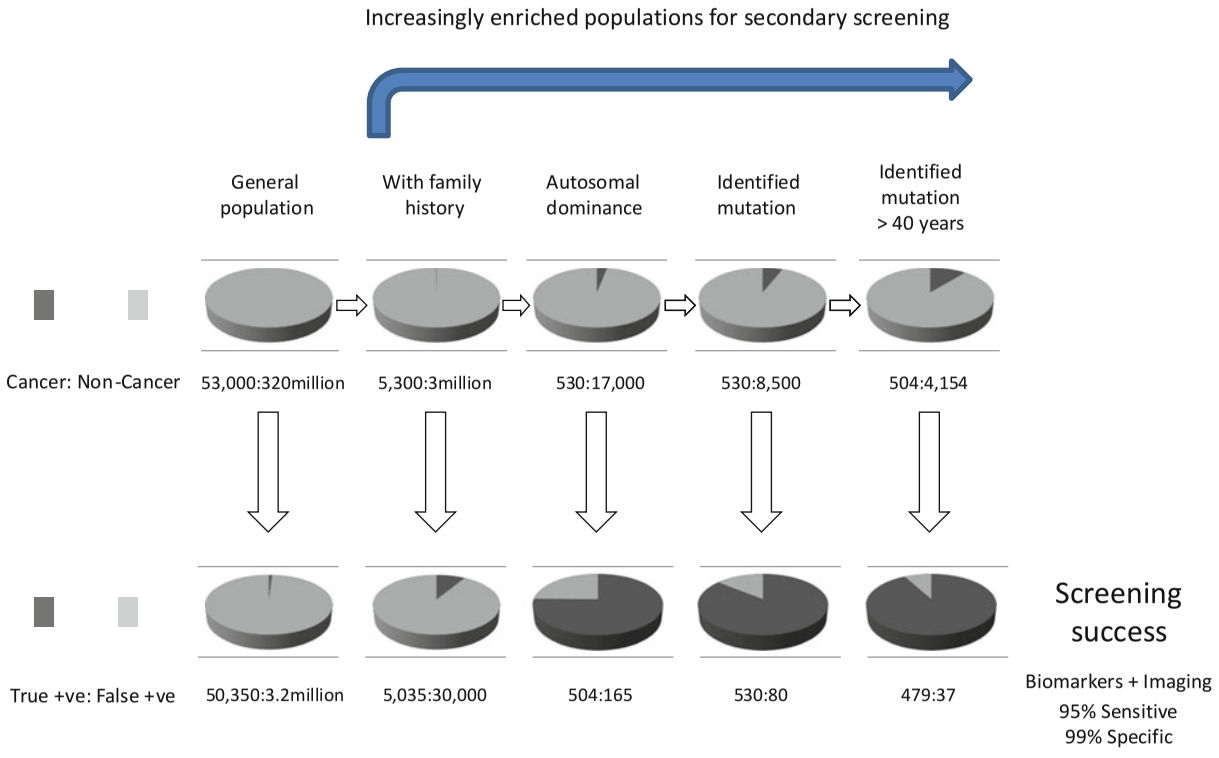

Fig. 2 Evaluation of primary and secondary screening. The upper set of pie chart shows how the proportion of pancreatic cancer cases in a population changes with primary screening. The rationale explaining these numbers is given in the main text. If a secondary screening program with 95% sensitivity and 99% specificity was applied at each point in this primary screening process, the proportion of true positives and false positives would be as shown in the second row of pie charts. The numbers underneath the pie charts are based on an initial population of 350 million individuals and 35,000 cases of pancreatic cancer (roughly equivalent to the population of the USA)

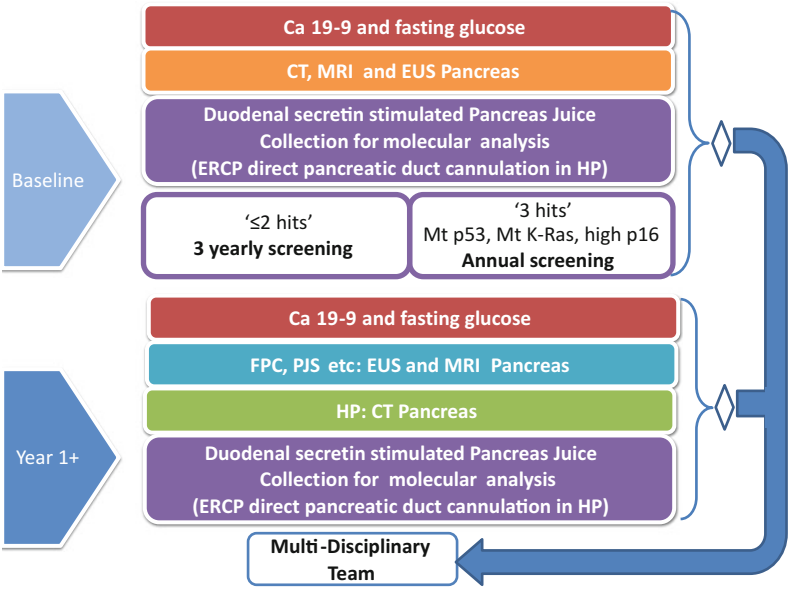

Fig. 3 The EUROPAC secondary screening protocol in FPC. Following identification of high-risk individuals by primary screening, members of FPC kindreds undergo baseline testing. If there is evidence of malignancy, the patient is referred to the clinical multidisciplinary team meeting (*MDT*). Otherwise, pancreatic juice is obtained by ERCP, and this juice is analyzed for molecular markers of cancer. If this analysis raises concerns, the patient is referred to the MDT. The MDT may suggest close surveillance which involves annual EUS and possibly ERCP, or they might recommend that the option of surgery is discussed with the participant. For those not under close surveillance, screening continues with a three yearly imaging cycle and ERCP for molecular analysis in the year following EUS. ◊ = clinical input)

First-degree relatives of a case of pancreatic cancer in a family with autosomal dominance would have a 50% chance of carrying the disease mutation; if this mutation could be identified, then the proportion of genuine at-risk individuals would be doubled.

Pancreatic cancer risk is heavily age dependent. If age is taken into account, then the proportion of individuals with cancer in the screened population can be greatly increased. Simply by limiting screening to people over the age of 40 years, the population without cancer will be roughly halved (median age in the USA is projected to be 40 years by 2025) with only a modest reduction in the number of cancer cases (<5%) [10].

The risk analysis will also have to take into account costs and morbidity associated with the secondary screening modalities. The question is therefore whether there are modalities available which offer adequate sensitivity and specificity at a reasonable cost.

Screening Modalities

Two main approaches have been employed to detect early pancreatic neoplasms and precursor lesions. The first is imaging predominantly with EUS, and the second relying on molecular analysis. Secondary screening requires higher specificity and sensitivity than is currently possible with any single modality, but combinations of imaging and biomarkers might be sufficient.

Imaging

Current imaging tests used in normal clinical practice for the investigation and management of pancreatic disease include; EUS, abdominal ultrasound (US), computed tomography (CT), magnetic resonance imaging (MRI), endoscopic retrograde cholangiopancreatography (ERCP), and ^{18}F-Fluorodeoxyglucose positron emission tomography (18FDG-PET).

CT, MRI, and EUS have all been employed in various combinations for the detection of early pancreatic neoplasms. A study in 2012 by Canto et al. undertook a prospective comparison of all three modalities in 225 asymptomatic IARs [43]. EUS detected a pancreatic abnormality in 43% of patients, in contrast with MRI and CT, which identified lesions in 33% and 11%, respectively. Five EUS detected lesions underwent surgical resection, of which three where intraductal papillary mucinous neoplasms (IPMN) with high-grade dysplasia.

EUS

A study from the CAPS Consortium showed that EUS had the highest detection rate of pancreatic abnormalities among high-risk patients (42.6%), compared to CT (11%) and MRI or magnetic resonance cholangiopancreatography (MRCP) (33.3%). EUS has also been shown to have a greater sensitivity and specificity than CT (>90% sensitivity [44]), particularly for small tumors [43]. Hanada et al. showed that for PDAC <10 mm, EUS was the most valuable imaging modality for tumor detection [45].

EUS is also particularly sensitive in detecting precursor cystic lesions of the pancreas such as IPMNs [46]. Other smaller pancreatic precursor lesions such as pancreatic intraepithelial neoplasia (PanIN) [47] are not directly visualized at EUS, but their presence might be inferred by pancreatic parenchymal heterogeneity and lobulocentric atrophy.

The accuracy and interobserver agreement of EUS is influenced by user experience, completion of advanced training, and the type of echoscope used. Radial echoendoscopes may miss some early lesions in patients with high risk for pancreatic cancer while linear scopes allow the operator the ability to perform fine needle aspiration (FNA) during the same procedure if an area of interest is identified making it more cost and time effective. The results of two large meta-analyses suggest that combining EUS and FNA to diagnose PDAC gives a high sensitivity (89–92%) and specificity (96%) [48, 50].

EUS however, is not very specific in patients with benign inflammatory diseases of the pancreas. Positive predictive value was only 60% in a study of 85 patients aimed at distinguishing between chronic pancreatitis and pancreatic cancer [50]. Combining EUS with FNA or "Tru-cut" biopsy does not fully resolve this problem as it is still very easy to miss small tumors in a patient with cancer in a background of chronic pancreatitis [51].

Overall, it would appear that EUS has several advantages over other screening modalities. If suspicious lesions are identified during the EUS, they can be sampled during the procedure, unlike with the other imaging methods and unlike CT, EUS does not expose patients to radiation or intravenous contrast. The procedural risk profile of EUS is like other endoscopic procedures and includes infection, bleeding, and perforation.

Computed Tomography (CT)

CT must use a pancreas specific protocol and requires a specialist radiologist to interpret the images. A retrospective analysis of CT scans showed that it was possible to pick up abnormalities up to 18 months before formal diagnosis with pancreatic cancer [52]. In practice tumors below 1 cm are almost impossible to detect [53]. Sensitivity is further reduced in the presence of chronic pancreatitis. CT has the added disadvantage that it involves exposing the participant to a dose of up to 10 millisieverts (mSv) of radiation for each scan [54]. Although it may be possible to reduce the dose depending on the size of the participant, the use of radiation in a screened population that could well include individuals with inherited DNA repair defects is a matter of concern.

Magnetic Resonance Imaging (MRI)

MRI is an alternative that does not involve the use of ionizing radiation. It has also been reported that use of high tesla magnets and T1 weighted spin-echo can give images of small lesions that are significantly better than those seen with spiral CT [44, 55]. MRI is more sensitive for identifying cystic lesions, and that MRI and EUS appear to be complementary rather than interchangeable [56–58].

Positron Emission Tomography (PET)

^{18}Fluorodeoxyglucose (18FDG) is not metabolized so will accumulate in all cells where there is high glucose uptake such as in primary tumors and metastases [44]. 18 FDG-PET is not very helpful in detecting early stage PDAC, which in one study identified only 50% of stage 0 and 1 tumors [59] and two of seven IPMNs [60], with care being taken to ensure normal blood glucose levels.

Endoscopic Retrograde Cholangiopancreatography (ERCP)

While ERCP is claimed to produce images that are characteristic of PanIN lesions, irregular or ectatic ducts with possible sacculations [47], this should not be used for imaging because of the unacceptable risk of complications [47]. ERCP can also be used to collect pancreatic juice for molecular analysis via direct cannulation of the pancreatic duct [61], but while the complications are very low in hereditary

pancreatitis they are unacceptably high in suspected familial pancreatic cancer with a normal parenchyma and main pancreatic duct [62]. The main complication is post-ERCP acute pancreatitis even with the use prophylaxis with nonsteroidal anti-inflammatories or self-expelled pancreatic stents [62]. The preferred method is pancreas juice obtained by secretin stimulation of the pancreas and duodenal aspiration at endoscopy [63]. More recently, pancreatic juice collected directly from the ampulla using an endoscopic distal cap yielded higher concentrations of pancreatic fluid mutations than form duodenal aspiration [64].

Defining Imaging Screening Success

Consensus agreement from the CAPS consortium was that for a screening program to be deemed successful, it should lead to the identification and treatment of T1 N0 M0 margin negative PDAC, and high-grade dysplastic precursor lesions such as PanIN Grade 3, main duct intraductal papillary mucinous neoplasm (MD-IPMN), or a branch duct IPMN (BD-IPMN) with high-grade dysplasia [13]. Potentially relevant lesions might also include multifocal PanIN2 (>10) lesions, BD-IPMNs with low- or moderate-grade dysplasia and/or atypical flat lesions, and pancreatic neuroendocrine tumors (pNETs) (>G0) [58].

Screening outcomes were reported on 411 asymptomatic high-risk individuals using annual MRI and or EUS from three European centers [65]. There were 214 individuals from FPC families, 178 CDKN2A mutation carriers, and 19 BRCA1/2 or PALB2 mutation carriers. PDAC was detected in 13 (7.3%) of 178 CDKN2A mutation carriers with a 75% resection rate and a 24% 5-year survival rate. Two CDKN2A mutation carriers (1%) underwent surgical resection for low-risk precursor lesions. Two individuals (0.9%) in the FPC cohort had a pancreatic tumor, including one advanced PDAC and one early grade 2 neuroendocrine tumor. Thirteen (6.1%) individuals with FPC underwent surgical resection for a suspected precursor lesions, but only four (1.9%) had high-risk lesions (high-grade IPMNs or grade 3 PanINs). One BRCA2 mutation carrier was found to have PDAC, and another BRCA2 mutation carrier and a PALB2 mutation carrier underwent surgery and were found to have low-risk precursor lesions. Surveillance of CDNK2A mutation carriers was relatively successful but the benefit of surveillance in families with FPC was less evident.

Biomarkers

Diabetes Mellitus (DM)

Long-standing DM increases the risk of PDAC by approximately twofold [66]. DM diagnosed shortly before diagnosis with PDAC is most likely caused by pancreatic cancer itself. Pancreatic cancer cells can produce prodiabetic substances such as adrenomedullin [67]. Approximately, 1% of patients diagnosed with type 2 DM will have newly diagnosed PDAC. New-onset DM in those over 50 years confers up to

an eightfold risk of having PDAC compared with the general population. Chari et al. have shown that the average time between the diagnosis of DM and the subsequent diagnosis of PDAC is 13 months [68]. Boursi et al. developed a clinical prediction model to assess risk for pancreatic cancer among patients with new-onset DM [69]. Data were analyzed from 109,385 patients with new-onset DM of whom 390 (0.4%) were diagnosed with PDAC within 3 years. The final model (area under the curve, 0.82; 95% CI, 0.75–0.89) included age, body mass index, change in body mass index, smoking, use of proton pump inhibitors, and antidiabetic medications, and levels of hemoglobin A1C, cholesterol, hemoglobin, creatinine, and alkaline phosphatase. If the predicted risk threshold for PDAC screening was set at 1% over 3 years, only 6.19% of the new-onset DM population would undergo screening, which would identify patients with PDAC with 44.7% sensitivity, 94.0% specificity, and a positive predictive value of 2.6%. New-onset DM in an FPC kindred would therefore be an early warning symptom of PDAC. Pilot screening studies have employed fasting glucose serum samples and serial HbA1c measurements.

Serum Antigens

CA19-9 is the only biomarker in routine use for the management and follow-up of PDAC with a sensitivity and specificity of ~85% [70, 71]. There is lack of expression in ~5% of the population (Lewis Le(a-b-) phenotype) and is elevated in chronic pancreatitis and obstructive jaundice [70, 72]. It may also be effective in the detection of early tumors and in asymptomatic individuals but with reduced sensitivity [73]. Other serum tumor markers such as carcinoembryonic antigen (CEA) and CA125 have a similar lack of sensitivity with small tumors but may be efficiently combined with novel biomarkers [74].

Circulating Tumor Cells (CTCs)

CTCs may be defined as cells isolated from blood with an intact nucleus, which stain positive for epithelial cell adhesion molecule (EpCAM) an epithelial cell marker, cytokeratin (CK) a marker of epithelial-derived cells and are negative for CD45 a universal marker of leukocytes. Detectable cancer cells have been found in the circulation of patients with advanced pancreatic cancer [75]. Despite these earlier promising reports, the detection rate CTCs and reproducibility in pancreatic cancer has remained challenging. Using the CellSearch (Veridex) system that exploits immunomagnetic capture with EpCAM, Kurihari et al. detected CTCs in seven of 24 patients with stage 4 pancreatic cancer with no false positives in 11 patients with chronic pancreatitis patients and 10 healthy controls [76].

Epithelial mesenchymal transition (EMT) is a central tenet involved in allowing for cancer cells to invade, disseminate, and metastasize [77]. Gorges et al. showed in murine breast cancer xenograft models that there was downregulation of EpCAM in CTCs (and hence not detected by Ep-CAM based methods), whereas mesenchymal markers like Twist and EGFR were upregulated on CTCs [78]. Khoja et al. compared CTC detection using CellSearch with isolation by size of epithelial tumor cells (ISET) that is a marker independent, blood filtration device in samples from

54 patients with pancreatic cancer [79]. ISET appeared to detect CTCs in more patients than CellSearch (93% vs. 40%) and in higher numbers with a median (range) 9 (0–240) versus 0 (0–144) CTCs per 7.5 ml. There was considerable CTC heterogeneity of expression for EpCAM, pan-CK, E-cadherin, vimentin, and CK7. While CTC concentration correlated with survival in the study by Kurihari et al. [78], there was no association in the latter study [79]. CTCs were not characterized by molecular analysis so the true numbers of actual CTCs isolated is not known.

Rhim et al. showed that EMT and dissemination preceded pancreatic tumor formation in a mouse model of pancreatic cancer. Tagged cells invaded and entered the bloodstream early, before any frank malignancy could be detected and was widely associated with EMT [77]. Circulating pancreatic cells maintained a mesenchymal phenotype, exhibited stem cell properties, and seeded the liver. EMT and invasiveness were most abundant at inflammatory foci, and induction of pancreatitis increased the number of circulating pancreatic cells, linking the increased risk of pancreatic cancer in sporadic and hereditary pancreatitis. Rhim et al. were subsequently able to detect circulating pancreas epithelial cells in patients with pancreatic cystic lesions [80]. Blood samples were analyzed using a geometrically enhanced differential immunocapture (GEDI) microfluidic platform with antibodies to EpCAM to capture circulating epithelial cells. Captured cells were then further characterized by staining with 4′, 6-diamidino-2-phenylindole (DAPI) to visualize nuclei and fluorescently conjugated antibodies to CD45, and CK19, a marker of epithelial-derived cells, or pancreas and duodenal homeobox protein-1 (Pdx-1). Thege et al. were able to further develop a microfluidic immunocapture system for capture of circulating pancreatic cells using parallel EpCAM and cancer-specific mucin 1 (MUC1) in a silicon microdevice [81]. They also detected a known oncogenic KRAS mutation in cells spiked in whole blood using immunocapture, RNA extraction, RT-PCR, and Sanger sequencing.

Huang et al. have gone on to show that dielectrophoresis has the potential to complement existing immunocapture techniques to improve capture performance of CTCs [78]. By carefully specifying the applied electric field frequency, they demonstrated that pancreatic cancer cells were attracted to immunocapture surfaces by positive dielectrophoresis whereas peripheral blood mononuclear cells were repelled by negative dielectrophoresis.

Circulating Free DNA (cfDNA)

Circulating free deoxyribonucleic acid has been used to screen for cancer [82], recurrence [83], and response to treatment [84]. Plasma contains approximately 1 μg ml of cfDNA [85], most of which is fragmented into multiples of 200 bp and comes from leukocytes and endothelial cells. In cancer patients, these levels can rise tenfold, during chemotherapy and radiotherapy [86]. Circulating free DNA is also elevated in many benign condition reducing specificity for cancer diagnosis [87]. Plasma DNA levels appear to decrease in acute pancreatitis [85]. A more promising approach is the detection of specific mutations in DNA isolated from plasma [88] and beyond that with next-generation sequencing.

Targeted Molecular Analysis

NGS or massively parallel sequencing methods can be applied very small amounts of DNA (ng levels), with prior amplification by PCR methods and fixed to nanobeads or enclosed in emulsion droplets. Deep Sequencing has been shown to be a viable approach to characterize circulating DNA in patients with diagnosed lung cancer [89] and screening of individuals at risk of sporadic retinoblastoma [90]. Yu et al. have used digital next-generation sequencing to detect low-abundance mutations in secretin-stimulated juice samples collected from the duodenum of subjects enrolled in Cancer of the Pancreas Screening studies at Johns Hopkins Hospital [91]. EUROPAC has also used NGS deep sequencing of Tp53 DNA isolated from pancreatic juice as part of its pilot screening program.

Prospectively, the flexibility of NGS may make it the technology of choice, but most research to date has been focused on identification of specific mutations in genes known to be associated with pancreatic cancer. Ideally, blood or even urine would be used as the source material, but pancreatic juice has the most intimate contact with the cells of the pancreatic ducts and so is the richest source of material from ductal cancers, PanIN and IPMN. Pancreatic cancer cells [92] and cells from IPMN [93] can be detected in pancreatic juice, but the number of cells is small even in advanced cancer [92].

Analysis of K-Ras Mutations

K-Ras mutations are the commonest mutations reported in pancreatic cancer [94] and are early events in pancreatic tumorigenesis. Over 90% of pancreatic cancers have been reported to have a mutation in codon 12 of the *K-Ras* gene [95] followed by mutations in codons 13 and 61 [96]. Sho et al. sued digital PCR (dPCR) to detect KRAS gene mutations in 44 pancreas FNAs including 34 formalin-fixed paraffin-embedded (FFPE) and 10 fresh samples [97]. The dPCR mutation analysis was successful in all preoperative FNA biopsies tested, and its accuracy was confirmed via comparison with resected tumor specimens. Moreover, dPCR revealed additional KRAS mutations representing minor subclones within a tumor that were not detected by Sanger sequencing. Maire et al. used Mutation Specific PCR (MSP) to detect K-Ras mutations in blood from PDAC patients [98] but similar mutations were also seen in patients with chronic pancreatitis and even healthy controls.

Trumper et al. achieved 33% sensitivity for pancreatic cancer using detection of *K-Ras* mutation in bile [99]. Similar levels of sensitivity have been reported using duodenal juice (25%) [100]. Specificity is poor with 7/93 patients with benign disease having mutation in bile [101] and 0/9 in duodenal juice [100].

Some groups have reported that detection of K-Ras in stool gives better sensitivity than bile, a huge advantage given a considerably less invasive procedure but with reduced specificity [102]. Work with prediagnosis stool samples from colorectal cancer patient suggests that molecular analysis is unlikely to give a great deal of discrimination from control patients [103], it is even less likely to be effective in patients with early pancreatic cancer.

K-Ras mutation analyses carried out in pancreatic juice gives sensitivity ranging from 32% to 89% and specificity from 33% to 100% [104]. Nearly all studies are that *K-Ras* mutations can be identified in pancreatic juice in patients with no evidence of malignancy, whether with chronic pancreatitis or other benign conditions such as biliary tract stones. There is however some discrimination for cancer patients and therefore the analysis might be useful in combination with other tests if the juice sample has already been collected for a more specific assay.

Analysis of Tp53 Mutations

Mutation specific approaches to analyze Tp53 mutations have been hampered by the existence of over 700 different pathological mutations, compared to seven common *K-Ras* mutations. Sequence specific approaches, such as MSP, would therefore only be possible with complex multiplex approaches [105]. Exosomes present in blood contain DNA, which can be amplified and sequenced directly; in this way, Tp53 along with *K-Ras* mutations were detected by simple Sanger sequencing [106].

Sensitivity of Tp53 mutations in pancreatic juice samples ranges from 14% to 60% with a specificity >80% [91] and Tp53 mutations have been reported in tissue from patients with chronic pancreatitis [107]. It has even proved possible to identify Tp53 mutations in duodenal aspirates by using a digital PCR approach combined with limiting dilution [108].

The Johns Hopkins Pancreas Cancer Screening program found that mutant Tp53/SMAD4 concentrations could distinguish PDAC from IPMN cases (AUC 0.73) and controls (AUC 0.82). Two of four patients who developed pancreatic cancer despite close surveillance had SMAD4/Tp53 mutations from their cancer detected in juice samples collected over 1 year prior to their pancreatic cancer diagnosis when no suspicious pancreatic lesions were detected by imaging [91].

Cancer-Associated Methylation

Although tumorigenesis is in general associated with a loss of DNA methylation, specific tumor suppressor genes are associated with increased silencing by methylation which can be detected by converting unprotected cytosine residues to uracil using bis-sulfite and then carrying out quantitative PCR using either primers specific for sequences containing cytosine (sequences protected by methylation) or uracil (unmethylated DNA), comparing the levels gives a measure of the degree of methylation. Alternatively, bis-sulphite treated DNA can be sequenced (typically pyrosequenced) revealing where cytosine residues have been protected. Cancer specific patterns of DNA methylation have been described in circulating plasma DNA [109], and Kisiel et al. [110] described a panel of methylated biomarkers (CD1D, KCNK12, CLEC11A, NDRG4, IKZF1, and PKRCB) which when used with K-Ras mutation analysis gave 75% sensitivity and 95% specificity comparing pancreatic cancer to normal pancreas and chronic pancreatitis.

Combination Testing of Molecular Markers in Pancreatic Juice

Yan et al. analyzed cell free pancreatic juice samples for *K-Ras* and Tp53 mutations combined with quantification of *CDKN2A* (p16) promoter methylation [61]. Functional p53 mutations were detected in 20/48 (42%) cases of pancreatic ductal adenocarcinoma. No p53 mutations were seen in 49 controls. Two p53 mutations were seen in 49 (4%) patients with chronic pancreatitis (4%). K-Ras mutations were seen in 31/57 (54%) of PDAC patients but also in 13/61 (21%) of controls and 23/67 (34%) of patients with chronic pancreatitis. Twenty out of 21 (95%) of PDAC patients had p16 promoter methylation levels above 0.1% compared to 6/22 (27%) of controls and 4/20 (20%) of patients with chronic pancreatitis; 13/20 (62%) of PDAC patients had p16 promoter methylation levels above 10% compared to 2/22 (9%) of controls and 3/20 (15%) of patients with chronic pancreatitis. A Bayesian analysis assuming a pretest probability of cancer of 1%, suggested that this approach could stratify risk of cancer between negligible and 90% in the case of FPC and between negligible and 50% in HP [61].

Wang et al. studied both *K-Ras* and Tp53 in combination, using DNA prepared from pellet and supernatant. By combining all results for Tp53 and *K-Ras,* they observed a mutation (either *K-Ras* or Tp53) in a sample (either pellet or supernatant) in 100% (21/21) of cancer cases [111]. Clearly, this demonstrates that some patients exhibit Tp53 mutations without *K-Ras* mutations, and that combination analyses are useful for enhancing the molecular diagnosis of pancreatic cancer [111], [112]. As Wang et al. observed no Tp53 mutations in their control group specificity was determined purely by their *K-Ras* results, it is unclear what specificity was obtained with a combination of results using cellular and using noncellular material [111].

Yu et al. undertook digital NGS assays using an Ion AmpliSeq Custom Panel to multiplex PCR and sequence of nine genes (122 amplicons in two primer pools) mutated in pancreatic ductal neoplasms (KRAS, GNAS, TP53, SMAD4, CDKN2A, RNF43, TGFBR2, BRAF, PIK3CA) [91]. Ninety-six aliquots of DNA from each patient's juice were made and each aliquot was subjected to NGS. The study was undertaken in 115 people with PDAC, IPMN), and controls. Cases with PDAC and IPMN were more likely to have mutant DNA detected in pancreatic juice than controls; mutant DNA concentrations were higher in patients with PDAC than IPMN or controls [91].

National Registries for FPC and High-Risk Individuals

The Purpose of Registries

A disease registry is a collaboration between researchers, consenting volunteer participants, and clinicians with a special interest in patients with the disease. Dedicated registries offer clear benefits for all stakeholders.

For participants on an FPC registry, the process of registration can include a realistic discussion to help clarify an individual's actual PDAC risk (often over

perceived by the individual), education on risk modification and increasing awareness of alarm or red flag symptoms. In addition, once registered, many participants feel they now have more credibility when discussing their concerns or fears with other clinicians, such as general practitioners for example. Registries may also be in a position to offer support for participants through patient/family networks.

Researchers will use information gathered from the registrants to develop a large, detailed epidemiological data set that can be interrogated to test and refine assumptions on the natural history of the disease. Registrants also offer researchers a proactive group of appropriate individuals on which to test the yield of various screening modalities as part of a secondary screening research study. Improvements in diagnostic modalities for detecting early pancreatic cancer have historically been hampered as the vast majority of research biological samples were obtained from individuals with advanced disease. Registries facilitate the opportunity for healthy participants to donate vital samples: blood, DNA, urine, and saliva for example. These samples, collected before diagnosis, may help in the development of novel biomarkers for early disease.

Registries are not however, without disadvantages: individuals with a family history of pancreatic cancer have a higher perceived risk of PDAC and higher levels of anxiety [113], and these concerns can be amplified by the process of registration and recruitment. Potentially, the most detrimental is the inevitable inclusion of false positive families and inappropriate individuals.

Pilot Studies on Screening of High-Risk Groups

There are numerous established International and National registries of FPC families and other high-risk groups in the USA, Europe, Canada, and Australia, and now newly established in Japan. Many of these registries are associated with screening programs or pilot studies. The published outcomes of various screening programs from groups including; Johns Hopkins Hospital (JHH), the University of Washington (UW), the Memorial Sloan-Kettering Centre (MSKC), the German National Case Collection for Familial Pancreatic Carcinoma (FaPaCa), the Spanish National Hereditary Pancreatic Cancer Registry (PanGen-FAM), the Danish national screening program and the Swedish screening program are summarized in Table 2. All these studies rely on EUS and MRI as screening modalities.

The NFPTR was the first registry in the world established at the Johns Hopkins by Ralph Hruban in 1994 and as of February 2013, they had recruited 1447 families with FPC. Groups in Europe closely followed such as the EUROPAC at Liverpool University established in 1996, and FaPaCa at Phillips University (Marburg, Germany) established in 1999.

Individuals recruited to NFPTR as part of the American Cancer of the Pancreas Screening (CAPS) Consortium. The first study in 2004 included 38 patients from FPC families and Peutz-Jeghers syndrome sufferers [47]. EUS and CT were the modalities of choice and ERCP was employed in the event of abnormalities detected by imaging. One invasive cancer was found along with an IPMN and two serous

Table 2 Screening programs for FPC and IARs for PDAC

Study	Number included	Program base	Duration of follow-up/ study period	PDAC	MD IPMN	Surgery	Other findings
Brentnall et al. [121][b]	14	UW (USA)	–	0	0	7	Dysplasia
Kimmey et al. [122][b]	46	UW (USA)	5 years	0	0	12	12 dysplasia
Canto et al. [47][b]	38	JHH (USA)	1998–2001	1		7	1 IPMN 2 SCA 3 PanIN 1–2 1 PanIN 3
Canto et al. [114][b]	227 (78 FPC/ PJS, 149 controls)	JHH (USA)	2001–2004	0	0	7	7 IPMN (1 progressed to advanced PDAC during FU) 1 PanIN
Langer et al. [116][b]	76	FaPaCa (Germany)	1999–2007	0	0	6	1 BD-IPMN 3 SCA 1 PanIN 2 1 PanIN 1
Poley et al. [123]	44	Netherlands	2005–2007	3[c]	0	10	7 BD-IPMN
Verna et al. [124]	51	Columbia (USA)	2005–2008	2	0	5	5 BD-IPMN 7 other cystic lesions
Ludwig et al. [125]	109	MSKCC (USA)	2002–2009	1	1[a]	6	2 BD-IPMN 1 SCA 1 PanIN3 1 PanIN2
Schneider et al. [117][b]	72	FaPaCa (Germany)	1999–2009	1	0	9	3 SCA 1 PanIN 3 2 IPMN 1 PanIN1/2
Vasen et al. [120]	79 FAMMM only	Netherlands	2000–2010 (median 4, range 0–10 years)	7 (3[c])	0	5	9 other cystic lesions
Zubarik et al. [126]	27	UVM (USA)	2006–2009	1	0	3	1 NET 1 PanIN1
Al-Sukhni et al. [127]	262	Canada	2003–2011 (av 4.2 years)	3		4	15 BD-IPMN 65 other cystic lesions 1 pNET
Canto et al. [43][b]	216	CAPS (USA)	Median 28.8 months (range, 14–47.2 months)	0	2	5	82 Cystic lesions 5 resected = 2 MD IPMN and 3 BD-IPMN) 3 pNET
Potjer et al. [128][b]	125 FPC	FaPaCa (Germany)	Median FU 34 months	1	1	11	51 other cystic lesions Including: 5 BD-IPMN with 4 PanIN 2–3,

(*continued*)

Table 2 (continued)

Study	Number included	Program base	Duration of follow-up/ study period	PDAC	MD IPMN	Surgery	Other findings
							1 PanIN 1 3 SCA 3 PanIN 1 only
Sud et al. [129]	30 (Inc PJS and BRCA2)	USA	2008–2011	2[c]		3	1 IPMN (LGD)
Mocci et al. [118]	41	PanGen-Fam (Spain)	2 years	0	0	1	1 pNET 1 PanIN 3
Joergensen et al. [119]	40 FPC (31 HP)	Danish national screening program (Netherlands)	2006–2014	2	–	2	
Harinck et al. [57]	139	Dutch research group on PC (Netherlands)	12 months	1		2 (PDAC, multifocal PanIN2)	9 cystic lesions 1 PanIN2
Del Chiaro et al. [56]	40	KUH (Sweden)	2010–2013 (mean 12.9 months)	3 (2[c])	2[c]	5 (3 PDAC, 2 IPMN)	9 BD-IPMN 3 mix type 1 IPMN (1 with PDAC)
Bartsch et al. [58]	253	FaPaCa, the Leiden and Madrid registry, (Germany)	2002–2015 Median 28 (1–152) months	2	0	21	1 BD-IPMN with HGD 3 SCA 1 pNET 5 PanIN 2–3 6 PanIN 2 with BD IPMN
Total	1780			30	6	131	23 PanIN2–3 2 pNET

pNET pancreatic neuroendocrine tumor
SCA serous cystadenoma
[a]Suspected but patient declined surgery
[b]Multiple publications from same study group with potential for duplication of patients
[c]Identified on baseline

cystadenomas (SCAs). The 2006 CAPS-2 study had 72 participants from FPC families and six had Peutz-Jeghers syndrome [114]. Sixty-seven participants had a spiral CT scan. At this time, ERCP was often employed as a screening modality and for collection of pancreatic juice. Sixty-four of these participants had successful ERCP, five of whom developed pancreatitis as a result. Suspected neoplastic lesions were identified in 17 cases. Of these, 10 continued with surveillance and seven proceeded to subtotal pancreatectomy, IPMN's and PanIN lesions but no cancers were found. One participant developed metastatic pancreatic cancer in an interval between screening [114]. The 2012 CAPS-3 study was a multicenter prospective cohort study [43] involving the Mayo Clinic (Rochester), University of California (Los Angeles), Dana Farber Cancer Institute (Boston), and MD Anderson Cancer Center (Houston). The majority of patients (195/216) in this study were from eligible FPC kindreds, 19 came from BRCA2 families and two had Peutz-Jeghers syndrome.

Patients were followed up for a mean of 28.8 months (range, 14–47.2) and the final diagnoses at study completion were confirmed or suspected BD-IPMN ($n = 82$), combined IPMN ($n = 2$), and pNET ($n = 3$). Pancreatectomy was performed on five individuals, none of whom had any evidence of invasive PDAC (one Whipple procedure, three distal, and one total pancreatectomy) and no major adverse events or mortality were reported [43].

The group from Washington University have also previously reported results for PDAC screening in75 patients, 15 had abnormalities on EUS and ERCP, all of whom had surgery (12 total and three distal pancreatectomy) [115]. The three that had distal pancreatectomy remain under surveillance. Histology revealed PanIN-3 lesions in 10 of the resected specimens and the remaining five contained PanIN-2. Although no cancers have yet been detected by screening, similarly to the JHH group, one participant of the screening program developed an unresectable pancreatic cancer in the interval between screens [115].

The FaPaCa registry is a national case collection for FPC families funded by the Deutsche Krebshilfe organization. Multiple centers have collected families with at least two first-degree relatives with confirmed PDAC since 1999, with the study being coordinated centrally by the Philipps-University of Marburg in Germany. The surveillance program has evolved following publication of the 5 year [116] and 10 year screening results [117] which demonstrated a relatively low diagnostic yield of potentially relevant lesions. Initial annual screening with MRCP and EUS was carried out between 2002 and 2010. From 2011, imaging consisted of annual MRI with MRCP and EUS every 3rd year or when suspicious alterations were detected by MRI. In collaboration with the Leiden and Madrid registries, between July 2002 and June 2015, 253 Caucasian IAR (210 Marburg, 30 Madrid, 13 Leiden) completed at least baseline imaging. The 253 IAR underwent a total of 813 MRI and 450 EUS, including FNA cytology in five IAR. Following MDT discussion, 21 IARs underwent pancreatic resection. Histopathological analysis identified six (2%) IAR with significant lesions, two with PDAC, (stages 1and 2b, respectively), three with PanIN3, and one with IPMN high-grade dysplasia [58]. Lesions were more often identified in IAR above the age of 45 years. In 21 IAR who underwent surgery, no clinically significant lesions were detected before the age of 50 years and potentially relevant lesions occurred significantly more often after the age of 50 years (13 and 2, respectively). The group concluded that it is safe to delay the start of screening in FPC kindreds until 50 years of age [58].

EUROPAC has a three yearly screening cycle, with EUS imaging at the end of each cycle, followed by collection of duodenal juice and molecular analysis the year after. In patients who did not have juice collection or who had a cancer-associated mutation in their duodenal juice, there was an annual pathway consisting of repeat blood testing and EUS. Any abnormalities identified in imaging or molecular tests were discussed at the supraregional pancreatic multidisciplinary team (MDT) meeting. The significance of positive molecular results is considered in the context of the age and perceived risk of the participant based on family history. The MDT may recommend further clinical investigations, advice surgery or propose that the participant undergoes annual surveillance or regular clinical review and/or follow-up.

The PanGen-Fam registry was established in Spain in 2009. Eligibility criteria include two or more cases of PDAC, Lynch syndrome with one case of PDAC in the family, melanoma and 1 PDAC case, Peutz-Jeghers syndrome, HP, and families with one case of PDAC occurring below 50 years of age. Screening consists of baseline imaging with CT and EUS; MRI is only performed if the latter are abnormal. Screening starts 10 years earlier than the youngest age at PDAC diagnosis in the family or at 40 years old, whichever comes first. For Peutz-Jeghers syndrome and HP families, screening starts at age 30 and at age 35 years, respectively. The follow-up frequency is stratified according to family risk and the results of previous tests. So far, screening has identified four cystic lesions and one pNET, and one patient who underwent resection had high-grade dysplasia [118].

The Swedish group based at Karolinska University Hospital includes members of FPC kindreds (as defined by the CAPS consortium), carriers of mutations in *BRCA2*, *BRCA1*, or *CDKN2A* with at least one first- or second-degree relative with PDAC, and verified germline carriers of a Peutz-Jeghers syndrome kindred [56]. This group performed a prospective observational study of 40 eligible participants with a genetic risk for PDAC who were referred between January 1, 2010, and January 31, 2013. All patients entered an MRI based screening protocol, where all participants underwent a baseline MRI/MRCP with secretin. If no abnormality was identified then additional screening was planned for 1 years' time. Patients only underwent an EUS with or without FNA if an abnormality was detected on MRI. The mean age of patients was 49.9 years and the mean length of follow-up was 12.9 months. The diagnostic yield for a pancreatic lesion including solid nodules, cysts, and isolated main duct dilation was 40%. Five patients (12.5%) required surgery, three (7.5%) for pancreatic cancer and two for IPMN with intermediate dysplasia. MRI revealed a pancreatic lesion in 16 patients (40%), an IPMN in 14 (35%) and PDAC in two (5%). One patient had a synchronous IPMN and PDAC [56].

The Dutch research group includes the Erasmus MC-University Medical Centre Rotterdam, the Academic Medical Centre Amsterdam, the University Medical Centre Groningen and the Netherlands Cancer Institute-Antoni van Leeuwenhoek Hospital [57]. They run an ongoing multicenter familial pancreatic cancer surveillance study where eligible asymptomatic individuals with an estimated $\geq$tenfold increased familial or inherited PDAC risk compared with the general population undergo screening from the age of 45 years or 10 years younger than the age of the youngest relative with PDAC, whichever occurred first. For patients with Peutz-Jeghers syndrome, the minimal age for inclusion is 30 years or 10 years younger than the age of the youngest relative with PDAC. Using their screening population, this group undertook a multicenter prospective blinded cohort study comparing EUS and MRI for the detection of clinically relevant pancreatic lesions at first-time screening in individuals at high risk for developing PDAC. This study identified two 9 mm solid lesions (one stage 1 PDAC and a multifocal PanIN2) both at EUS, and nine cysts $\geq$10 mm, in nine of the high-risk individuals screened (6%). Of the cystic lesions, six were detected both by EUS and MRI and three were detected by MRI

only [57]. These results support the current consensus that EUS is good at detecting small solid lesions and MRI is very sensitive at detecting cystic lesions.

A cost effectiveness study for screening for PDAC in IARs from the Netherlands has estimated the incremental cost-utility ratio (ICER) for screening. The ICER for patients with FPC was estimated at 28,834 US$ per life-year and 38,785 US$ per Quality Adjusted Life Years (QALY) [119].

Conclusion

Pilot studies of pancreatic cancer screening in high-risk individuals have shown that it is feasible but cost effectiveness remains challenging. The issues are the low yield of cancers in the screened population and the rate of interval cancers. The detection rates range from seven cancers in 79 participants [120] to no cancers in 227 participants [114]. The difference in yield is not explained by differences in screening modalities or frequency, as the approaches were very similar. The rate of screening failures (inoperable cancers) was similar in programs with high yields as in programs with low yields. The difference is therefore in cancer prevalence in the screened populations. This indicates that improving risk stratification to allow more targeted screening is essential. Further research is also warranted on how screening uptake can be optimized in the highest risk groups.

Given the low number of cancers so far detected by screening, it is difficult to make any firm conclusion about the best protocol for detecting early PDAC. The most commonly detected lesions are IPMN and following surgery, PanIN lesions. This supports the use of MRI for detection of cystic lesions and EUS for detecting small solid lesions. EUS however will struggle to detect early pancreatic cancer against a background of chronic pancreatitis, so other modalities and strict adherence to the screening protocol is required.

Text Boxes

Key Practice Points

- It is important to ask every pancreatic cancer patient about family history and to take anxiety about family history seriously.
- The finding of two cancer cases in a family is not enough to confirm an inherited predisposition. The number of cases must be placed in the context of the number of family members who did not develop cancer and look at the generations involved to determine phase of transmission.
- Where a family history is of concern, referral to a pilot screening study is appropriate. The protocol should take into account age and likely risk of individuals in the family before accepting the referral.

Published Guidelines

Consensus recommendations for secondary screening of high-risk groups were proposed at the Fourth International Symposium on Inherited Diseases of the Pancreas [13]. The following categories of high-risk individual were considered appropriate for inclusion in a research based screening program.

- Anyone with >2 first-degree, second-degree, or third-degree relatives with pancreatic cancer in the same lineage.
- Any known mutation carrier for *BRCA1*, *BRCA2, PALB,* or *CDKN2A* (p16), with at least one first-degree or second-degree relative with pancreatic cancer.
- A person with PJS.
- Mismatch repair gene mutation carriers (Lynch syndrome) with one affected first-degree relative.
- Anyone with two relatives in the same lineage (directly connected) affected with pancreatic cancer, at least one a first-degree relative of the candidate.
- An affected individual with hereditary pancreatitis, harboring a PRSS1 mutation.

Future Research Directions

- Selecting high-risk groups that are likely to benefit from screening.
- Identifying efficient and cost effective screening programs.
- Improvement in accuracy of existing biomarkers.
- New biomarkers and new combinations of biomarkers.
- Improved imaging modalities, such as novel imaging compounds for use with PET.

Cross-References

- ▶ Circulating Tumor Cells
- ▶ Clinical Decision-Making in Pancreatic Cancer
- ▶ Development of Novel Diagnostic Pancreatic Tumor Biomarkers
- ▶ Diagnostic Biomarkers
- ▶ Epidemiology and Prospects for Prevention of Pancreatic Cancer
- ▶ EUS and Its Role in Pancreatic Cancer
- ▶ Cancer Exosomes for Early Pancreatic Cancer Diagnosis and Role in Metastasis
- ▶ Familial Pancreatic Cancer
- ▶ Management of Cystic Neoplasms of the Pancreas Including IPMNs
- ▶ The Molecular Pathology of Precursor Lesions of Pancreatic Cancer
- ▶ MRI and MRCP for Diagnosis and Staging of Pancreatic Cancer
- ▶ Pancreatic Adenocarcinoma: CT and PET/CT
- ▶ Paraneoplastic Syndromes in Pancreatic Cancer

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Role of Radiotherapy in Locally Advanced Pancreatic Cancer

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Contents

Abstract

Pancreatic cancer carries a poor prognosis regardless of stage, and incidence and death rates are increasing. Pancreatic cancer is divided into four general categories, resectable, borderline resectable, locally advanced/unresectable, and

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metastatic. Only 15–20% of patients diagnosed with pancreatic cancer have resectable or borderline resectable disease at diagnosis. Most patients are diagnosed with more advanced disease; approximately 30–40% of patients present with locally advanced, unresectable pancreatic cancer (LAPC) at the time of diagnosis, and another 40% have distant metastatic disease. Surgery provides the only chance of cure for patients with pancreatic cancer, but the likelihood of patients with unresectable disease ultimately proceeding to surgical resection is low. The management of these patients with locally advanced, unresectable disease is controversial, and there is no internationally accepted regimen. The data for the use of radiation therapy in the setting of LAPC will be discussed in this chapter.

Keywords

Pancreatic cancer · Radiation therapy · Chemoradiation · Hypofractionation · Stereotactic body radiotherapy (SBRT) · Particle therapy

Introduction

Pancreatic cancer carries a poor prognosis regardless of stage, and incidence and death rates are increasing. An estimated 53,070 pancreatic cancer diagnoses and 41,780 pancreatic deaths are projected for 2016 [1]. Pancreatic cancer is divided into four general categories, resectable, borderline resectable, locally advanced/unresectable, and metastatic. Only 15–20% of patients diagnosed with pancreatic cancer have resectable or borderline resectable disease at diagnosis. Despite potentially curative resection, the 5-year survival for patients undergoing pancreaticoduodonectomy is 10–20% [2]. Most patients are diagnosed with more advanced disease; approximately 30–40% of patients present with locally advanced, unresectable pancreatic cancer (LAPC) at the time of diagnosis, and another 40% have distant metastatic disease. Median survival in patients with locally advanced disease is 12–13 months [3], and in patients with metastatic disease at presentation, survival is approximately 6–11 months [4, 5].

Determination of resectability is based on computed tomography (CT) scan, magnetic resonance imaging (MRI), and endoscopic ultrasound (EUS) data collected at diagnosis. As surgical techniques are refined, the categorization of patients similarly changes. At present, LAPC is defined by encasement of more than 180 degrees around the superior mesenteric artery (SMA), celiac artery, or aorta, unreconstructable superior mesenteric vein (SMV), or occlusion of the SMV. There are emerging data to suggest that reconstruction of the celiac artery is technically feasible and safe, but this is a novel approach that is not widely practiced [6–10].

Surgery provides the only chance of cure for patients with pancreatic cancer, but the likelihood of patients with unresectable disease ultimately proceeding to surgical resection is low. The management of these patients with locally advanced, unresectable disease is controversial, and there is no internationally accepted

regimen. The data for the use of radiation therapy in the setting of LAPC will be discussed.

Radiation Alone Versus Chemoradiation

An early clinical trial addressing chemoradiation for LAPC was published in 1969 from Mayo Clinic and included patients with various types of GI cancers, 64 of whom had locally unresectable pancreatic cancer. These patients were randomized to either 5-fluoruracil (5-FU) or placebo, combined with 35–40 Gy radiation [11]. Median survival in the combined modality arm was significantly higher than in the radiation therapy-only arm (10.4 vs. 6.3 months, $p < 0.05$). The Gastrointestinal Tumor Study Group (GITSG) subsequently randomized 194 patients with locally advanced pancreatic cancer to receive split-course external beam radiation therapy (EBRT), either alone (60 Gy) or combined (40 or 60 Gy) with bolus 5-FU. The EBRT-alone arm was discontinued after an interim analysis demonstrated superior median time to progression and overall survival in the combined modality arms. One-year survival was 11% in the EBRT-alone group compared to 38% and 36% with 40 and 60 Gy, respectively. No significant differences were seen between high- and low-dose EBRT in the chemoradiation arms, although there were trends favoring the higher-dose arm in time to progression and survival [12, 13]. Thus, these two randomized studies demonstrated a modest survival benefit for combined modality therapy over EBRT alone.

A more modern clinical trial from the Eastern Cooperative Oncology Group (ECOG) also examined the question of radiation therapy alone versus concurrent chemoradiation. In this study, 114 patients were randomly assigned to EBRT alone (59.4 Gy) or the same EBRT regimen plus infusional 5-FU and mitomycin C (MMC). In contrast to the above trials, the concurrent chemoradiation arm was noted to have increased toxicity without any added disease-free survival or overall survival benefit. Likely contributing to this finding was the method of administration and dosing of 5-FU. In the prior GITSG study, 5-FU was administered as 500 mg/m^2 on the first 3 days of each course of 20 Gy radiation. For the ECOG study, 5-FU dosing was extrapolated from other gastrointestinal disease sites and given at 1,000 mg/m^2 daily on days 2 through 5 and 28–31. Additionally, mitomycin C was added to this already high-dose infusional regimen and likely further contributed to toxicity [14]. In a pooled analysis of both the GITSG and ECOG studies, in spite of the added toxicity from the ECOG chemotherapy regimen, chemoradiation increased survival over radiotherapy alone (hazard ratio [HR] for death 0.69, 95% CI 0.51–0.94) [15] (Table 1).

Chemotherapy Alone Versus Chemoradiation

The above data suggest that chemoradiotherapy is superior to radiation alone, but do not address whether concurrent chemoradiation provides significant benefit over chemotherapy alone for patients with locally advanced disease. Two trials, also from

Table 1 Prospective studies comparing radiation alone versus chemoradiation for LAPC

Trial	Intervention	Number of patients	Local recurrence	Disease-free survival (mo)	Overall survival (mo)	Acute toxicity, grade 3+	Late toxicity, grade 2+
Mayo Clinic 1969	RT + placebo	32	NR	NR	6.3	NR	NR
	RT + 5-FU	32			10.3		
					$p < 0.05$		
GITSG 1981	RT (60 Gy)	25	24%	2.9	5.3	NR	NR
	RT (40 Gy) + 5-FU	83	26%	7.0	9.7		
	RT (60 Gy) + 5-FU	86	27%	7.6	9.3		
			NS	$p < 0.01$[a]	$p < 0.01$[a]		
ECOG 2005	RT	49	NR	5.0	7.1	25%	NR
	RT + 5-FU/ MMC	55		5.1	8.4	33%	
				NS	NS		

NR not reported, *NS* not significant

[a]Significant difference between RT alone and CRT (chemoradiation) arms; no difference between CRT arms

GITSG and ECOG, investigated this question in the 1980s. The GITSG study randomized 43 patients with locally unresectable pancreatic cancer to receive SMF (streptozocin, mitomycin C, and 5-FU) alone for 2 years or SMF chemotherapy with 54 Gy radiation followed by additional SMF chemotherapy. The trial was closed early due to poor accrual, but there was a statistically significant survival benefit to chemoradiation over chemotherapy alone (41% vs. 19%, $p < 0.02$) at the cost of increased toxicity in the CRT arm [16]. The ECOG trial randomized patients with unresectable gastric and pancreatic adenocarcinoma to receive either 5-FU alone or 40 Gy radiation with concurrent 5-FU. Of the 91 pancreatic cancer patients enrolled in the study, there was no difference in time to recurrence or overall survival in the chemotherapy alone versus chemoradiotherapy groups. Toxicity was significantly higher in the chemoradiation arm (27% vs. 51%, $p < 0.02$), but radiation dose at 40 Gy in 20 fractions was lower than modern treatment prescriptions [17].

The introduction of gemcitabine and the recognition of benefit in patients with metastatic disease stimulated the design of trials comparing gemcitabine to contemporary chemoradiation approaches with conflicting outcomes. The Fédération Francophone de Cancérologie Digestive (FFCD) and the Société Francophone de Radiothérapie Oncologique (SFRO) conducted a trial examining gemcitabine alone versus 60 Gy radiation plus concurrent 5-FU and cisplatin chemotherapy followed by gemcitabine alone. With 119 patients, there was a statistically significant survival advantage to gemcitabine alone over chemoradiation (13 months vs. 8.6 months, $p = 0.03$). Notably, a median survival greater than 10 months had not previously been reported in a multi-institutional phase II or phase III trial evaluating chemotherapy alone for this stage of disease. The acute toxicity in the chemoradiation arm was high leading to poor compliance, with only 42.4% of patients receiving at least 75% of the intended dose of chemoradiation, which likely contributed to the poor outcomes for the cohort. The median number of maintenance gemcitabine infusions was significantly less in the patients treated with chemoradiation as opposed to gemcitabine alone (6 vs. 10), and the median total dose of gemcitabine was also significantly less (6,845 vs. 15,000 mg/m^2). Factors that likely contributed to the poor tolerance were the inclusion of cisplatin, the high dose of radiation (60 Gy), and the treatment of regional nodes (larger treatment fields compared to treatment of just tumor and involved nodes) [18].

In a more recent study by Loehrer et al., also examining the use of gemcitabine with radiation, 74 patients were randomized to gemcitabine (600 mg/m^2 weekly) with radiation (50.4 Gy in 28 fractions to primary disease and regional nodes) followed by weekly gemcitabine (1,000 mg/m^2 weekly, for 3 of 4 weeks) versus gemcitabine alone (1,000 mg/m^2 weekly, for 3 of 4 weeks). Although the trial closed prematurely after accruing only 74 of a planned 316 patients, a statistically significant median survival benefit was seen in the arm that received chemoradiation compared to the arm that received chemotherapy alone, 11.0 versus 9.2 months, ($p = 0.017$). This benefit came at the cost of increased acute gastrointestinal toxicity (grade 3 or greater gastrointestinal toxicity 38% vs. 14%, $p = 0.03$). Additionally, overall grade 4 or greater toxicity was higher in the chemoradiation arm as compared to gemcitabine alone (41% vs. 9%). Thus, the addition of radiation to standard

Table 2 Prospective studies comparing chemotherapy alone versus chemoradiation for pancreatic cancer

Trial	Intervention	Number of patients	Local failure	Overall survival (mo)	Acute toxicity, grade 3+	Late toxicity, grade 2+
GITSG 1988	CRT (5-FU + SMF)	22	45%	9.7	NR	NR
	SMF	21	48%	7.4		
			NS	$p < 0.02$		
ECOG 1985	CRT (5-FU)	47	32%	8.3	NR	NR
	5-FU	44	32%	8.2		
			NS	NS		
FFCD/ SFRO 2008	CRT (5-FU/ CDDP)	59	64%	8.6	65.5%	NR
	Gemcitabine	60	72%	13	40%	
				$p = 0.03$	$p = 0.008$	
ECOG 2011	CRT (gemcitabine)	34	12%	11	82%	NR
	Gemcitabine	37	30%	9.2	80%	
			NS	$p = 0.017$		

NR not reported, *NS* not significant, *CCDP* cisplatin

chemotherapy resulted in a modest prolongation of median survival at the cost of acute toxicity. Notably, however, there were no differences in health-related quality of life outcomes between the two treatment groups beyond week 6, with long-term measurements taken at week 15 or 16 and at 9 months [19] (Table 2).

Induction Chemotherapy Prior to Chemoradiation

Given the lack of a consistently demonstrated survival benefit to upfront chemoradiation in patients with LAPC, differing treatment algorithms have been explored. Approximately one-third of patients with LAPC develop metastatic disease during initial treatment [20, 21]. To allow for the selection of patients without micrometastatic disease who would benefit from local therapy, studies have examined the use of upfront chemotherapy followed by chemoradiation for those patients who do not develop progressive disease.

A retrospective series of 181 patients with LAPC treated with gemcitabine-based chemotherapy as part of phase II and III trials by the European Groupe Cooperateur Multidisciplinaireen Oncologie (GERCOR) explored the question of upfront chemotherapy. Chemotherapy was given alone for 3 months in each of the various protocols; the decision to give concurrent chemoradiation or continue chemotherapy alone for patients with locally advanced disease without evidence of disease progression was as per protocol or at the discretion of the treating physician. In each of the studies, the concurrent chemoradiation regimen was 55 Gy of radiation

therapy with concurrent infusional 5-FU. Of the 128 patients that did not develop metastatic disease following upfront chemotherapy, 72 patients completed a course of chemoradiation, while 56 continued to chemotherapy alone. Analysis of their outcomes revealed significant improvement in progression-free survival with chemoradiation over chemotherapy alone (10.8 months vs. 7.4 months, $p = 0.005$) as well as overall survival (15 months vs. 11.7 months, $p = 0.0009$) [20]. While the retrospective nature of this study limited the broad acceptance of this approach, the results were hypothesis generating.

In a subsequent phase III study by Hammel et al. examining the utility of initial chemotherapy alone followed by chemoradiation, patients were randomized in a 2×2 factorial design to gemcitabine with or without erlotinib for 4 months followed by a second randomization for patients that did not develop progressive disease to two additional months of gemcitabine chemotherapy or chemoradiotherapy. Chemoradiation consisted of 54 Gy radiation therapy with concomitant capecitabine dosed at 1,600 mg/m^2 daily. Radiation fields included the primary tumor and nodal disease with margin with no prophylactic treatment to uninvolved nodal regions. While locoregional progression was less frequent (32% vs. 46%, $p = 0.04$) and delay to treatment reintroduction was longer in the chemoradiation arm as compared to the chemotherapy alone arm (6.1 months vs. 3.7 months, $p = 0.02$), at a median follow-up of 36.7 months, there was no overall survival difference (15.2 months vs. 16.5 months, $p = 0.83$) [3]. Potentially contributing to the shortcomings of the chemoradiotherapy arm were significant radiation protocol deviations; 50% of patients had minor protocol deviations and 18% had major deviations. Although these deviations were not found to be statistically significant, there was a trend toward poorer outcomes for the patients with major deviations, such that overall survival from first randomization was 17 months for patients with per protocol and minor deviations versus 13.4 months for patients with major deviations (HR, 1.43; 95% CI, 0.86–2.36; $p = 0.17$). Median survival from the date of the second randomization was 12.7 months for patients with per protocol and minor deviations versus 10.1 months for patients with major deviations ($p = 0.19$). An additional limitation of the study is the choice of chemotherapy, as the trial was designed prior to the routine use of FOLFIRINOX or nab-paclitaxel plus gemcitabine combination therapies, which have now been shown to be more efficacious in metastatic disease compared to gemcitabine monotherapy [4, 5].

Choice of Concurrent Chemotherapy

The recent LAP07 study discussed above did not demonstrate a survival benefit to chemoradiation over chemotherapy alone. While these data may influence clinical practice, many patients with locally advanced disease still ultimately undergo chemoradiation for a variety of reasons, including palliation. Consequently, choice of concurrent chemotherapy is relevant.

Early trials investigating various concurrent chemotherapy options include the GITSG study as well as a study from Taipei. In the study by the GITSG, 143 patients

were randomized to either radiation therapy with concurrent 5-FU or radiation with concurrent doxorubicin. Radiation was delivered to total dose of 60 Gy in a split-course regimen and included elective nodal volumes. Concurrent 5-FU was delivered at 500 mg/m^2 on the first 3 days of each course of radiation therapy. Overall survival was higher in patients treated with radiation plus concomitant 5-FU (8.5 months vs. 7.6 months), leading to acceptance of concurrent 5-FU as standard of care [22]. A study conducted by Li et al. compared radiation delivered with either concurrent 5-FU or gemcitabine. Radiation was given to gross disease as well as elective nodal basins to a total of 50.4–61.2 Gy; concurrent chemotherapy with 5-FU was dosed at 500 mg/m^2 and delivered for the first 3 days every 2 weeks, and concurrent gemcitabine dosing was 600 mg/m^2/wk. Patients that received concurrent gemcitabine had a statistically significant improvement in median overall survival (14.5 months vs. 6.7 months, $p = 0.027$) without increase in toxicity relative to the patients that received concurrent 5-FU. Additionally, patients in the radiation therapy plus gemcitabine arm had significant improvement in pain control, quality of life, and Karnofsky Performance Status (KPS) as compared to the 5-FU arm without significant increase in grade 3 or higher toxicity [23]. In spite of these findings suggesting that radiation with concurrent gemcitabine may be more efficacious as compared to concurrent 5-FU, this regimen has not been universally adopted, as this was a single-institution study with small patient numbers.

A more recent randomized phase II study, the SCALOP trial, explored the optimal concurrent systemic therapy by comparing radiation with concurrent gemcitabine versus capecitabine. Extrapolating data from other gastrointestinal malignancies, such as rectal cancer, led to the acceptance of concurrent capecitabine as equivalent to infusional 5-FU [24]. This has been further substantiated by uncontrolled trials that have used capecitabine in place of infusional 5-FU in patients with LAPC [25, 26]. In the SCALOP regimen, 74 patients with unresectable disease were treated with initial gemcitabine and capecitabine for 12 weeks (dosed at 1,000 mg/m^2 and 830 mg/m^2, respectively), and if patients did not develop progressive disease, they proceeded to chemoradiation with either concurrent gemcitabine or concurrent capecitabine. Both chemoradiation arms received 39.6 Gy to gross disease and involved regional nodes plus additional 10.8 Gy boost to the gross disease. There was no elective nodal irradiation. The primary endpoint of the trial was progression-free survival; at 9 months of follow-up, there was no statistically significant difference in this endpoint. Although not powered for survival, there was a statistically significant difference in median overall survival, which was 15.2 months in the capecitabine group and 13.4 months in the gemcitabine group (HR 0.39, $p = 0.012$). More patients in the gemcitabine group had grade 3–4 hematologic toxicity as compared to the capecitabine group, though non-hematologic toxicity was not significantly different. Additionally, quality of life metrics were not different between the two groups [27]. While the SCALOP trial may suggest apparent increased efficacy of 5-FU-based regimen over gemcitabine, this study should be interpreted carefully. The rationale for using gemcitabine with concurrent chemotherapy was to exploit the increased radiosensitization properties of gemcitabine as compared to 5-FU; however, the systemic contribution of

gemcitabine must also be considered. In the trial from Taipei, the gemcitabine delivered concurrently with radiation was dosed at 600 mg/m^2/week. The SCALOP study used only 300 mg/m^2/week of gemcitabine concurrent with radiation. While the patients in the SCALOP trial had previously received systemic doses of gemcitabine for 12 weeks prior to chemoradiation, this dose reduction during concurrent treatment could have contributed to the difference in outcomes between the study arms.

Role of Radiation Dose and Treatment Volumes

Despite efforts to improve outcomes for patients with LAPC with various novel chemotherapy regimens, induction chemotherapy, and concurrent chemoradiation, survival remains poor. Multiple studies have examined the use of hypofractionated radiation, intraoperative radiation therapy (IORT), image-guided radiation therapy (IGRT), intensity-modulated radiation therapy (IMRT), and stereotactic body radiotherapy (SBRT) as a means of increasing dose to the target tissue with hope that these techniques will improve outcomes.

Hypofractionation with Conventional Treatment

Dose escalation using conventionally fractionated regimens (1.8–2.0 Gy per fraction) was previously explored and did not demonstrate improvement in outcomes. The GITSG study, published in 1979, discussed previously, randomized patients to radiation with or without concurrent chemotherapy. Patients that received radiation alone were treated to a total of 60 Gy at 2 Gy per fraction in a split-course regimen. This regimen was compared to two other split-course chemoradiation arms, one that received 40 Gy radiation at 2 Gy per fraction and one that received 60 Gy at 2 Gy per fraction. As noted earlier, the radiation alone arm was discontinued early after interim analysis revealed inferior outcomes. Comparison of the two chemoradiation regimens revealed comparable outcomes in the 40 Gy and 60 Gy arms, suggesting potential for increased toxicity with dose escalation without added survival benefit [12].

More recently, new interest in hypofractionation has emerged, particularly in the setting of technologic advances that allow for more accurate targeting of both tumor and normal tissue. While this addresses the same basic hypothesis as the GITSG study, that increased dose should lead to improved local control and potentially improved survival, it also raises the issue of patient convenience. A retrospective review from the University of Texas at San Antonio and MD Anderson Cancer Center compared outcomes of patients treated concurrently with 5-FU and either 30 Gy radiation or more than 30 Gy radiation. Of the 107 patients included, 86 had been treated with 30 Gy in 10 fractions and 18 patients had been treated with 50.4 Gy in 28 fractions. There was no difference in local disease progression, development of distant metastasis, or overall survival for the two groups. Twenty-nine percent of the

high-dose group and 12% of the low-dose group were hospitalized for grade 3 treatment-related toxicity ($p = 0.05$) [28]. The results suggest that a shorter, hypofractionated regimen may be better tolerated than standard fractionation with similar rates of local and distant disease progression and equivalent median survival.

A prospective trial from Germany was performed for patients with either locally advanced or metastatic pancreatic cancer exploring various hypofractionated regimens with concurrent 5-FU chemotherapy. In this trial, patients were treated with 24 Gy in 8 fractions, 30 Gy in 10 fractions, or 36 Gy in 12 fractions. Grade 3 toxicity was seen in one patient. For the 20 patients with locally advanced disease included in this study, the median survival was 9 months, which is similar to survival times seen at the time of publication of that study in 2005 [29].

Intraoperative Radiation Therapy (IORT)

Although there is newfound interest in hypofractionation and dose escalation, the concept of hypofractionation, delivering large doses per fraction, has been a part of clinical practice in pancreatic cancer for decades. IORT is a long-standing technique that allows for the administration of a single, high-dose radiation treatment to areas of tumor involvement while sparing normal tissue by physically displacing or shielding organs at risk. Although not widely practiced, largely due to lack of randomized evidence and the invasiveness of the approach, IORT can provide reasonable rates of local control. A retrospective study of 194 patients with LAPC treated with IORT from Massachusetts General Hospital reported 2-year progression-free survival rates of 41%. Median overall survival was 12 months [30]. While encouraging, careful patient selection may have contributed to these numbers. Outside clinical trials, IORT should only be reserved for highly selected patients at institutions experienced at IORT for LAPC.

Intensity-Modulated Radiation Therapy (IMRT) and Image-Guided Radiation Therapy (IGRT)

Outside of the operating room, surrounding critical structures within the upper abdomen have limited the extent of dose escalation. Newer radiation techniques, including IMRT and IGRT, have allowed for dose escalation both with conventionally fractionated treatment and with hypofractionated therapies while respecting normal tissue tolerances. IMRT is a general term used to refer to a group of technologies that allow very conformal radiation fields to be delivered by altering the intensity of the beam. This is achieved by breaking the beam up into many smaller "beamlets." IMRT was a natural progression from the rapid increase in computing power applied to radiation treatment planning. With the advent of virtual simulation using CT scan and image reconstruction, the predecessor of IMRT, three-dimensional conformal radiotherapy (3D-CRT), gained wide use. This allowed

"beam's eye views" to be generated and radiation portals to be shaped to conform to tumor volumes and avoid critical structures. IMRT built on this concept by adding a multi-leaf collimator, where multiple leaves are able to slide in and out of the field to create a vast array of complex radiation portals. This can be either a dynamic process (leaves sliding while the beam is on) or static process (leaves slide into place, then dose is delivered). As a result of multiple leaf positions, or control points, the intensity of a beam from a single portal can be variable, rather than fixed. A combination of such modulated beams from different angles is used to produce the final dose.

During the treatment planning process, critical structures are identified and assigned dose constraints. The computationally intensive process of inverse planning using sophisticated software then respects these constraints while delivering the intended dose to the target. The result is a much more conformal radiation dose to target. This conformality is generated at the expense of increased low-dose exposure to a greater tissue volume. In part, this is due to the limitations imposed by the physics of photons, which deposit energy at the target, but also can generate a substantial exit dose.

The feasibility of dose escalation in pancreatic cancer has been explored in several dosimetric studies using IMRT. One study compared dose escalation of plans using 3D-CRT to two different kinds of IMRT, one where the boost was integrated and another where the boost was delivered sequentially [31]. In these plans, the dose was escalated from 54 to 64.8 Gy. The 3D-CRT plan often exceeded tolerance doses for normal tissues, including the small bowel, spinal cord, and liver, while both IMRT plans allowed for successful dose escalation without exceeding tissue tolerance and allowed for a reduced volume of the kidney receiving 20 Gy.

Another group conducted a dosimetric feasibility study using a dose optimization technique called generalized equivalent uniform dose (gEUD) [32]. They examined escalation of dose to the planning treatment volume (PTV, tumor with margin to cover microscopic disease and setup error). They also examined escalating the dose to the vascular margin, which is the margin that is most often involved following surgical resection and the site that generally precludes margin-negative surgical resection. These investigators demonstrated the feasibility of escalating the dose to the PTV from 52 to 66 Gy and to the vascular margin to as high as 85 Gy without exceeding tolerance dose to critical structures.

In a retrospective study by Ben-Josef and colleagues, 15 patients with pancreatic cancer (7 of whom had locally advanced, unresectable disease) were treated to a total of 61.2 Gy at 1.8 Gy per fraction to the gross tumor volume and 45 Gy to the surrounding lymph node basin. All patients received concurrent capecitabine. Overall, treatment was tolerated well with one patient developing grade 3 toxicity (gastric ulceration with bleeding that responded to medical therapy). In patients with unresectable disease, the 1-year actuarial survival was 69%. Conversion to resectability occurred in two patients, and these patients continued to be locally controlled at the time of study publication. Upon comparison of dose-volume histograms (DVHs) of IMRT versus standard 3D-CRT plans, the IMRT plans were noted to be more

conformal than the 3D-CRT plans. The median volumes of the small bowel receiving greater than 50 Gy or 60 Gy were reduced with IMRT over 3D-CRT treatment, and on normal tissue complication probability models, the small bowel complication probability was 9.3% with IMRT versus 24.4% with 3D radiation ($p = 0.021$) [33]. Though this study suggests that IMRT is a reasonable treatment method for patients with unresectable pancreatic cancer, it is limited by the fact that the median dose actually delivered was only 54 Gy, not 61.2 Gy, the study intended dose.

A study from the University of Chicago reported the outcome of 25 patients with pancreatic and bile duct tumors treated with IMRT plans [34]. In a subset of the patients, the IMRT plans were compared to conventional four-field 3D-CRT. The treatment was well tolerated, with 80% experiencing grade 2 or less acute GI toxicity. Median follow-up was 10.2 months. Only four patients experienced late grade 1 toxicity and one patient experienced late grade 4 toxicity. Comparison to 3D-CRT demonstrated a significant reduction of mean dose to the liver, kidneys, stomach, and small bowel.

In a subsequent prospective study from Ben-Josef et al., dose escalation using IMRT in conjunction with gemcitabine was explored. While the previous studies suggested that dose escalation with IMRT was safe and feasible, improvement in survival due to this intensification of local treatment was not shown. Given its enhanced radiosensitizing properties, concurrent gemcitabine along with increasing radiation dose was hypothesized to improve local control. The primary endpoint of the study was to identify the radiation dose associated with dose-limiting toxicity, defined as grade 3 or higher gastrointestinal (GI) toxicity, neutropenic fever, or deterioration of performance status to greater than or equal to 3 in 25% of patients. Patients were treated with induction and concurrent gemcitabine dosed at 1,000 mg/m^2on days 1 and 8 of each 21-day cycle. Radiation was delivered to the gross disease plus a 1 cm margin with escalating doses from 50 to 60 Gy, all in 25 fractions delivered over the course of 5 weeks. Fifty patients were included in the study, and dose-limiting toxicity was seen in 11 patients, including seven patients with grade 3 or 4 GI toxicity (nausea, vomiting, anorexia, or dehydration), three patients with duodenal bleed, and one patient with duodenal perforation. Two of the toxicities were seen at the 52.5 Gy dose, six were seen at 55 Gy, and three were at 57.5 Gy. Dose-limiting toxicity was felt to be reached at 57.5 Gy; the recommended dose was 55 Gy with a probability of a dose-limiting toxicity of 0.24. The 2-year freedom from local progression was 59% (95% CI 32–79) and median survival was 14.8 months, which was encouraging compared to historical control findings with median survival of 11.2 months at the time of publication [35]. Following treatment, 12 patients ultimately underwent surgery, 10 of which were margin negative; median overall survival for those that did undergo surgery was 32 months [36].

Providing further evidence that dose escalation can improve outcomes in patients with LAPC is a recent study from MD Anderson. In this retrospective review, 200 patients with LAPC with tumors greater than 1 cm away from luminal organs were treated with escalated doses of IMRT. The median dose delivered was 50.4 Gy, though there was a wide range of doses with various fractionation regimens. Dose escalation was achieved via a boost delivered to the gross disease plus a 2–5 mm

margin. All patients were treated with induction chemotherapy with either gemcitabine-based regimens or FOLFIRINOX in addition to concurrent chemotherapy with either gemcitabine- or capecitabine-based regimens. Radiation doses were subsequently compared using biologically effective doses (BED), calculated using the equation below, where n is the number of fractions, d is the dose per fraction, and α/β for tumors is 10:

$$\mathrm{BED} = nd\left[1 + \frac{d}{\alpha/\beta}\right]$$

The BED for a standard fractionation regimen of 50.4 Gy in 28 fractions is 59.47 Gy, and the BED for a dose-escalated and hypofractionated regimen of 57.25 Gy in 25 fractions is 70.36 Gy. Patients were stratified into low- and high-dose groups based on BED $<$70 Gy or BED $\geq$ 70 Gy. At a median follow-up of 9.6 months, patients in the high-dose group had superior overall survival as compared to the low-dose group (17.8 vs. 15.0 months, $p = 0.03$). Local-regional recurrence-free survival was also improved in the high-dose group (10.2 vs. 6.2 months, $p = 0.05$). There was no additional toxicity in the high-dose group [37].

Stereotactic Body Radiation Therapy (SBRT)

The modest increase in dose achieved with IMRT has been shown to be feasible and improve outcomes, but is still delivered over multiple daily fractions. Further dose increases had previously been limited by surrounding normal tissue tolerances. In order to deliver high, biologically effective doses of radiation therapy without damaging surrounding organs, more targeted delivery of radiation therapy was developed. This is termed stereotactic body radiation therapy (SBRT). Stereotactic refers to a technique for precisely directing a medical instrument or beam of radiation in three planes using coordinates provided by medical imaging with treatment delivered in one to five treatments. Stereotactic brain biopsy and stereotactic radiosurgery to the brain have been widely used by implementing an externally fixed frame to ensure precision. Early stereotactic radiosurgery to extracranial sites made use of a stereotactic body frame [38]. Since organ motion in the region of the pancreas can be significant, these early systems employed some form of motion management. More recent advances in extracranial stereotactic body radiotherapy have used internal and externally placed fiducials. Other image-guided stereotactic radiosurgery systems were subsequently developed, including the CyberKnife, which consists of a small linear accelerator mounted on a highly flexible robotic arm with six degrees of freedom. Such machines can direct beams of radiation from hundreds of different angles toward the target and produce a highly conformal treatment. These treatment machines can also image radiopaque fiducial markers placed in or near the tumor to account for intrafraction tumor motion [39]. The most recent versions of this technology have the ability to track the tumor during treatment, which is accomplished by placing external fiducial markers and correlating

their position relative to internal fiducial markers [40]. The improved accuracy in targeting that is gained through the various stereotactic techniques allows for a significant reduction in the margins normally given to the tumor. These reduced margins allow for tolerable toxicity profiles even in the context of higher, ablative doses of radiation. Extracranial stereotactic radiotherapy has been successfully employed in several different sites, including primary and metastatic liver and lung tumors [41], and several studies have been conducted to examine its feasibility and efficacy in pancreatic cancer.

Stereotactic radiosurgery for LAPC was evaluated in a phase I dose escalation study conducted at Stanford University [42]. A CyberKnife system was used with internally placed fiducials, and a breath-hold technique was employed. Dose was escalated from 15 to 25 Gy to determine maximum tolerated dose. The target volume received a maximum dose as high as 41.6 Gy. The mean dose to 50% and 5% of the duodenum was 14.5 and 22.5 Gy, respectively. The treatment was well tolerated. No patient receiving 25 Gy had grade 3 or greater toxicity. All patients included in the 25 Gy group had local control of their pancreatic tumors until death or at last follow-up. Despite these high local control rates, all patients experienced distant progression. In an effort to improve on the median survival of 8.0 months seen at the 25 Gy dose level, the same group of investigators later conducted a phase II trial using 45 Gy of IMRT with concurrent 5-FU followed by a stereotactic boost of 25 Gy. Although high rates of local control (94% 1-year freedom from local recurrence with median follow-up of 23 weeks) were again seen, the median survival in this group of 16 patients was only 33 weeks, largely due to distant disease progression. Additionally, higher toxicity was observed, with two of the 16 patients treated experiencing grade 3 GI toxicity (gastroparesis requiring parenteral management). The next report from the Stanford group included a group of 16 patients treated on a phase I trial that combined gemcitabine with SBRT in an effort to address both local and distant disease. In this study, patients achieved a median survival of 11.4 months with 50% of patients alive at 1 year; however, these results came at the cost of high rates of late GI toxicity, with five patients developing duodenal ulcers (grade 2), one patient developing duodenal stenosis (grade 3), and one duodenal perforation (grade 4) [43].

A subsequent phase II study was performed at Stanford. Twenty patients with LAPC were treated with 25 Gy delivered in a single fraction with priority given to meeting duodenal constraints [44]. The dose to 5% of the duodenum was limited to 22.5 Gy, and 50% of the volume of the duodenum could receive a maximum of 12.5 Gy. Treatment was delivered with linear accelerator (linac)-based SBRT with IMRT. PTV margins were 2–5 mm. One-year local control was 94%, and toxicity was comparable to conventionally fractionated chemoradiation, with a single grade 4 toxicity (duodenal perforation, 5%) and three grade 2 toxicities (duodenal ulceration, 15%) [45, 46].

Fractionated SBRT regimens have also been investigated in prospective studies. A linac-based SBRT regimen with 45 Gy delivered in three fractions was examined in a multi-institutional phase II study from Denmark. Twenty-two patients were enrolled on the study, and at 1 year of follow-up, local control rates were poor at 57%. Additionally, rates of toxicity were high, with 79% of patients experiencing

grade 2+ acute toxicity. Potentially accounting for the increased toxicity was the use of abdominal compression, which can move the duodenum and bowel closer to the target volume, inclusion of peritumoral edema in the target volumes, resulting in larger volumes, and larger PTV margins (5 mm in the transverse and 10 mm cranio-caudal directions) than those used in the Stanford regimens [47].

More recently, a fractionated SBRT regimen was explored in a multi-institutional prospective phase II trial of 49 patients from Johns Hopkins, Stanford, and Memorial Sloan-Kettering [48]. The primary endpoint of this study was rate of grade 2 toxicity with a fractionated SBRT regimen [43]. Prior to receiving SBRT, patients were treated with up to three cycles of gemcitabine; radiation was then delivered to a total of 33 Gy in five fractions with central review of treatment plans. This study had strict requirements, including fiducial marker placement, respiratory motion management, and stringent dose constraints. Similar to preceding data, there was a high rate of local control (79% at 1 year), and median overall survival was 13.9 months. A lower rate of toxicity was reported with this multi-fraction regimen versus single-fraction treatment, with one acute (2%) and three late grade 3+ GI toxicities (6%). A final report of the quality of life endpoints has not yet been published, but in a review, the authors noted no decline in quality of life with improvement in pain (Table 3) [49].

Particle Beam Therapy

Protons and other particles, including helium, neon, and carbon ions, behave differently in tissue than photon radiation. While photons deliver their energy relatively superficially with a gradual dose falloff, proton beams penetrate tissue to variable depths depending on energy; this energy is then deposited in a sharp peak, known as the Bragg peak. The rapid falloff of energy at a prespecified depth limits exit dose and energy transfer to surrounding normal tissues. Theoretical models suggest that the Bragg peak may result in decreased rates of toxicity with particle therapy, though clinical data, particularly in pancreatic cancer, are limited.

In a study by Hsiung-Stripp et al., two- and three-field proton treatment plans were compared dosimetrically to conformal X-ray plans for patients with LAPC. While tumor coverage was not different for the two treatment modalities, doses to critical organs, including the liver, kidneys, and spinal cord, were significantly lower with the proton plans. Doses to the duodenum were not explored [50]. A similar study by Zurlo et al. compared proton plans with five- and nine-field IMRT plans for two patients with LAPC. Dose constraints to the kidneys, liver, and small intestine were met with proton plans, but were not able to be met with the IMRT plans [51]. Although these studies suggest that proton treatment may be superior to photon therapy with regard to normal tissue toxicity, both 3D-CRT plans and IMRT plans are easily able to meet dose contraints with conventionally fractionated treatment in clinical practice. The main concern is dose to the duodenum, particularly in the setting of dose escalation. Given the proximity of the duodenum to the gross disease in LAPC, it has been hypothesized that by exploiting the Bragg peak, proton therapy should allow for decreased duodenal

Table 3 Prospective studies of stereotactic body radiation therapy for pancreatic cancer

Study	Intervention	Number of patients	Local control (1 year)	Median survival (mo)	Acute toxicity, grade 3+	Late toxicity, grade 2+
Koong 2004 [42]	15–25 Gy/1 fx	15 LA or LR	100%	11	0%	NR
Koong 2005 [71]	45 Gy IMRT +5-FU	16	94%	8.3	13%	NR
	25 Gy/1 fx	LA				
Hoyer 2005 [47]	15 Gy × 3	22 LA	57%	5.4	79% grade 2+	94%
Schellenberg 2008 [43]	Gemcitabine	16	100%	11.4	6%	47%
	25 Gy/1 fx	LA				
	Gemcitabine					
Polistina 2010 [67]	10 Gy × 3	23 LA	50%	10.6	0%	0%
Schellenberg 2011 [44]	Gemcitabine	20	94%	11.8	5%	20%
	25 Gy/1 fx	LA				
	Gemcitabine					
Tozzi 2013 [69]	Gemcitabine	30	77%	11	0%	0%
	45 Gy/6 f. or 36 Gy/6 fx	LA or LR				
Gurka 2013 [68]	Gemcitabine	10	40%	12.2	0%	0%
	25 Gy/5 fx	LA				
	Gemcitabine					
Herman 2015 [48]	Gemcitabine	49	78%	13.9	12%	11%
	33 Gy/5 fx	LA				

NR not reported, *LA* locally advanced, *LR* locally recurrent

dose. While patient numbers were small, Kozak et al. compared conventionally fractionated photon treatment with 1.8 Gy delivered over 28 fractions to hypofractionated proton therapy with 25 CGE (cobalt gray equivalent) delivered over five fractions in nine patients. Mean doses to the duodenum were not statistically different between the two treatment types, suggesting that the dosimetric advantage of protons in the setting of LAPC may be limited [52].

A randomized trial comparing X-rays and helium ions was conducted at the Lawrence Berkeley Laboratory for patients with unresectable pancreatic tumors [53]. An RBE of 1.2 was assigned to the helium ion doses. RBE refers to the relative biological effectiveness of a particle beam, defined as the ratio of dose delivered to tumor cell kill, and allows for dose comparisons between photon and particle beam therapy. Patients were randomized to 60 Gy with split-course X-ray therapy concurrent with 5-FU or non-split-course 60–70 Gy equivalent (GyE) dose with helium ions concurrent with 5-FU. In the 49 evaluable patients, local control was estimated to be 10% in the helium-treated patients and 5% in the X-ray-treated patients

(p = not significant). Median survival was similar between the two groups (7.8 months in the helium-treated patients and 6.5 months in the photon group), and there were no significant differences in local control or metastasis-free survival. "Moderate-to-severe" gastrointestinal toxicity was seen in 33% of the helium-treated patients and 24% of those treated with photons.

The Heavy Ion Medical Accelerator group conducted early clinical investigations into the use of carbon ion treatment of patients with locally advanced as well as resectable pancreatic tumors [54]. Patients with unresectable disease were treated with doses escalated as high as 48 GyE in 12 fractions. Local control at 1 year was 81% and 1-year survival was 44% [55]. A more contemporary phase I/II study from the same group explored the use of carbon ion treatment with concurrent gemcitabine. The trial escalated both carbon ion doses from 43.2 to 55.2 GyE in 12 fractions with concurrent gemcitabine escalated from 400 to 1,000 mg/m^2. Dose-limiting toxicity was observed in 3 of the 76 patients enrolled (grade 3 infection in one and grade 4 neutropenia in two patients). One patient experienced a late grade 3 ulcer. Two-year freedom from local progression was 83%. Two-year overall survival rates were 35% for all patients and 48% in the high-dose group of patients without metastatic disease [56].

Radiation Treatment Planning Considerations

Simulation

Simulation technique varies depending on the selected radiation treatment modality. For patients undergoing non-SBRT therapy, either with 3D-CRT or IMRT, patients are typically imaged in supine position on an indexed wingboard or Vac-Lok bag (CIVCO Medical Solutions, Coralville, IA) with arms up. A CT scan is then performed with oral and IV contrast, which allows for better delineation of target and normal structures. A four-dimensional computed tomography (4DCT) can be helpful to assess the magnitude of tumor motion with normal respiration. For conventionally fractionated treatment, assessment of respiratory motion can also be achieved with fluoroscopic simulation. In this situation, patients are given oral contrast, and continuous X-ray beam images are taken with the treatment fields projected onto the imager to ensure that the treatment field completely encompasses the target throughout the respiratory cycle.

SBRT delivers highly conformal and precise radiation and requires additional imaging and technical considerations. Prior to simulation, fiducial markers are typically placed via endoscopic ultrasound in the tumor or in close proximity to the tumor; markers assist in target identification at simulation and treatment. Immobilization of patients is crucial for SBRT, and multiple different devices can be used, including an upper Vac-Lok (CIVCO Medical Solutions, Coralville, IA) on an indexed wingboard, Alpha Cradle (Smithers Medical Products, North Canton, OH), or BodyFIX (Elekta Instrument, Stockholm, Sweden). To account for respiratory motion, a four-dimensional computed tomography (4DCT) can be used.

Management of respiratory motion is more challenging with abdominal tumors than in other disease sites. For abdominal tumors, intrafraction and interfraction variation in target location due to respiration can be as much as 2–3 cm [57]. Given the precise nature of SBRT treatment, if motion exceeds 3–5 mm, respiratory motion management should incorporate active breathing control (ABC), breath-hold (preferably in end-expiration), or gating breath holding. There are data to suggest that end-expiration may allow for the most favorable anatomy to maximize therapeutic ratio [58]. Although abdominal compression is sometimes implemented to limit respiratory-related motion, this method has the potential to displace the duodenum and bowel toward the target volumes. Simulation imaging is then performed with a CT +/− MRI in the supine position with arms up. Both oral and IV contrast are generally used. At certain institutions, dual-phase IV contrast imaging is used, which allows for acquisition of arterial and portal venous phase images timed by bolus tracking. The use of multiphasic imaging allows for better delineation of the tumor, as pancreatic lesions are usually best seen in the portal venous phase [59]. Prior studies have suggested a relationship between pre-SBRT PET findings and clinical outcomes; some institutions perform a PET/CT simulation and utilize this for tumor response assessment post-SBRT [60, 61].

Definition of Treatment Volumes

Regardless of treatment modality, gross tumor volume (GTV) is identified based on simulation CT/MRI/PET and any other available diagnostic imaging. With conventionally fractionated treatment, a clinical target volume (CTV) is created, which includes nodal basins at risk for microscopic disease spread. These typically include the peripancreatic, pancreaticoduodenal, and portocaval nodes as well as nodal regions surrounding the celiac axis and superior mesenteric artery (SMA); splenic hilum nodes are often included for pancreatic tail lesions. The CTV is typically expanded by 5–10 mm to arrive at the PTV, which accounts for daily setup uncertainty.

Given the high doses that are used with SBRT, the treatment volumes for these hypofractionated regimens are much smaller. With multiphasic imaging performed for SBRT treatment planning, the GTV for each phase includes the primary tumor and potentially involved nodal disease. The GTV contours delineated in the various contrast phases are then combined to generate an internal target volume (ITV), which should be comparable to tumor motion identified on 4DCT. A PTV is then created based on a 2–3 mm expansion of the ITV, with consideration of adjacent normal structures, including the duodenum, stomach, and bowel, and additional coverage added as necessary, often in the retroperitoneum and along the vasculature. With proximity of the PTV to normal organs, selective underdosing of the PTV may be required to respect normal tissue constraints. Identification of an ITV for fiducial markers may also provide an additional reference for improved target identification during treatment delivery.

Treatment Planning

Conventionally fractionated radiation to the pancreas is delivered with 3D-CRT or IMRT. Typically 3D-CRT plans consist of opposed AP-PA beams with either opposed lateral or off-cord oblique beams to create a four-field approach. For a tumor in the pancreatic head, in order to ensure coverage of the nodal basins described above, the superior margin of the AP-PA fields should start around 1 cm superior to the porta hepatis, and the inferior border should lie around L2 or L3 to cover the entirety of the C-loop of the duodenum, depending on individual anatomy. The medial edge of the field should be shaped with an MLC (multi-leaf collimator) such that there is an approximate 1 cm margin along the C-loop of the duodenum. The lateral edge should be shaped to include an approximate 1 cm margin on the vertebral body to ensure inclusion of retroperitoneal nodal basins. The opposed lateral or oblique fields should have the same superior and inferior borders as the AP-PA fields; the posterior edge of the field should generally split the vertebral body; and the anterior field should allow for around 2 cm distance from the GTV. To avoid excess dose to normal tissue, the majority of the dose should enter through the AP-PA fields, while only 10–14 Gy should be delivered through the lateral or oblique fields.

SBRT benefits from the use of IMRT or VMAT to allow for delivery of ablative doses to the target while sparing normal tissue. More recently, flattening filter-free SBRT treatments have been explored, which allows for shorter treatment duration [62]. As discussed previously, the main concern with delivery of high doses of radiation per fraction is toxicity to the surrounding structures, particularly the duodenum. With single-fraction SBRT regimens used at Stanford, patients receiving 25 Gy in a single fraction had a higher rate of duodenal toxicity correlating with V15, V20, and Dmax (dose maximum, often normalized to 100%) [44]. With the transition to a multi-fraction regimen (33 Gy delivered in five fractions), a lower rate of toxicity was seen, as noted above [48]. The normal tissue constraints from this study serve as reasonable guidelines: the proximal duodenum (within 1 cm above and below the PTV), stomach, and small bowel with 9 cc $<$15 Gy, 3 cc $<$20 Gy, and 1 cc $<$33 Gy, the liver 50% $<$12 Gy, both kidneys 75% $<$12 Gy, and spinal cord 1 cc $<$8 Gy. Typical dose distributions for 3D-CRT, IMRT, and SBRT plans can be seen below (Figs. 1 and 2).

Treatment Delivery

Regardless of radiation treatment approach, image-guided radiation therapy (IGRT) to verify the target and normal tissues prior to delivery of treatment is recommended. This can be accomplished with a number of different techniques, including kV or fluoroscopic onboard imaging as well as cone beam CT (CBCT) just prior to treatment. Typically daily CBCT is used with SBRT, but there are data to suggest that CBCT may underestimate the abdominal motion of pancreatic lesions. Therefore, CBCT should be used to evaluate the location of normal tissue, and fluoroscopic

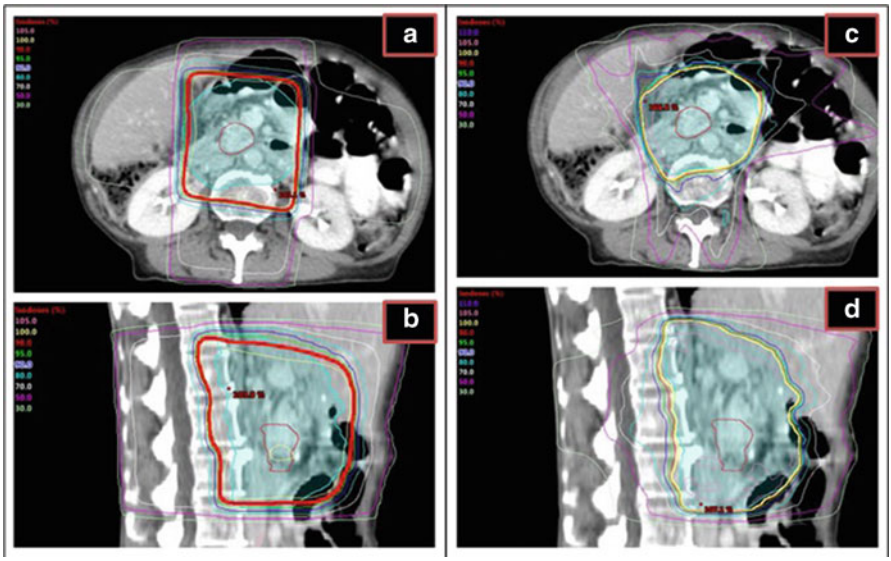

Fig. 1 3D-CRT and IMRT treatment plans. Dose distributions with external beam 3D-CRT and IMRT technique for LAPC. (**a**) 3D dose distribution, axial image. (**b**) 3D dose distribution, sagittal image. (**c**) IMRT dose distribution, axial image. (**d**) IMRT dose distribution, sagittal image. *Cyan*: PTV; *red*: ITV

images evaluating the alignment of fiducial markers should be used to complement CBCT information [57].

Future Directions

The high propensity of metastatic spread seen with pancreatic cancer and deaths related to metastatic disease limits the value of intensive local therapies. As systemic treatments improve, however, locoregional control will become increasingly important, and continuing efforts to optimize radiotherapy delivery are worthwhile.

A phase II trial from the Radiation Therapy Oncology Group (RTOG) attempted to address both the question of intensifying systemic therapy and local therapy in an effort to improve overall survival in patients with locally advanced disease. Unfortunately, the study recently closed due to poor accrual. Patients in the study were treated with neoadjuvant chemotherapy with gemcitabine plus nab-paclitaxel followed by intensified chemoradiation to 63 Gy in 28 fractions with concurrent capecitabine [63].

Given the low overall survival of patients with LAPC and high rates of distant progression, even in the setting of local disease control, more effective combined modality treatments are needed. A phase III study from Stanford is currently examining the safety and efficacy of a chemotherapy regimen known as modified

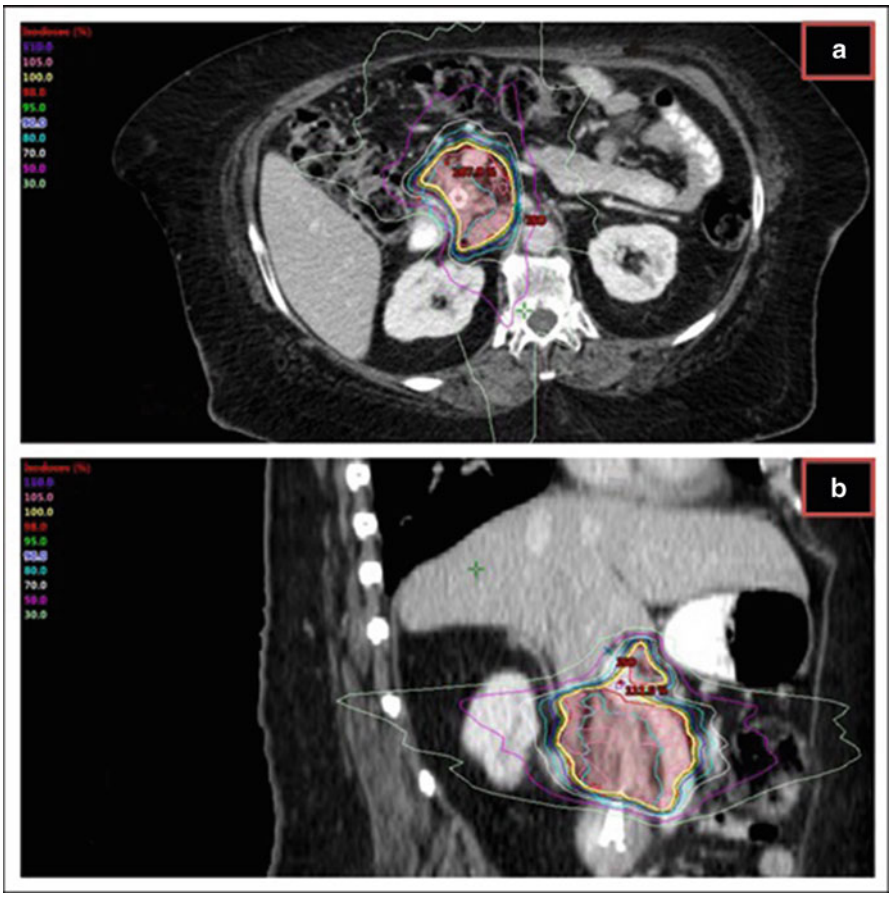

Fig. 2 SBRT treatment plan. Dose distribution with external beam SBRT technique for LAPC. (**a**) Axial image. (**b**) Sagittal image. *Red*, PTV; *cyan*, ITV

FOLFIRINOX (mFFX) alone or with the addition of SBRT in patients with LAPC [64].

Beyond chemotherapy, there is also an increasing interest in the role of immune therapies, with ongoing studies of whole cell tumor vaccines [65]. SBRT has also spurred much interest as a potential complement in the form of immunosensitization in many disease sites and may further enhance these systemic therapies [66].

Conclusion

Surgery remains the only option for cure for patients with pancreatic cancer. Approximately 40% of patients with pancreatic cancer present with locally advanced disease. The optimal management of these patients remains controversial, and there

is no internationally accepted regimen. Ideally, patients with locally advanced, unresectable disease should be treated on clinical trial. For most, an initial period of chemotherapy is appropriate. The period of upfront chemotherapy allows for selection of patients without development of overt metastatic disease; patients who do not progress through upfront chemotherapy may be appropriate for a number of subsequent regimens, including chemoradiation, continuing chemotherapy alone, or SBRT. While it is reasonable to reassess patients for resectability following these treatments, conversion to resectable disease is rare and the frequency of a complete resection with long-term survival is low. However, with advancements in radiation therapy as well as systemic therapy, including the use of more targeted and immune therapies, outcomes for patients with LAPC are likely to improve.

Cross-References

- ▶ Adjuvant Chemoradiation Therapy for Pancreatic Cancer
- ▶ Adjuvant Chemotherapy in Pancreatic Cancer
- ▶ Chemotherapy for Advanced Pancreatic Cancer
- ▶ Clinical Decision-Making in Pancreatic Cancer
- ▶ Neoadjuvant Chemotherapy in Pancreatic Cancer

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Vaccine Therapy and Immunotherapy for Pancreatic Cancer

Lei Zheng and Elizabeth M. Jaffee

Contents

Abstract

Recent advances in the tumor immunology field of research have enriched our knowledge of how tumor cells initially evade immune surveillance and how existing tumors actively suppress immune recognition of their progression. Based on these advances, strategies for immunotherapy have been developed to

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enhance antitumor immunity and to target the mechanisms underlying tumor evasion and immune tolerance. These immunotherapy strategies have been employed in the design of novel treatments for pancreatic cancer and are being tested in preclinical studies and human clinical trials. Evidence of immune activation has been demonstrated in a number of these studies and, in some cases, correlated with clinical responses. However, a number of challenges must be addressed before the true potential of immune-based therapies can be determined. Consequently, future studies need to focus on identifying new pancreatic cancer-associated antigens and on identifying and targeting the immune checkpoints that inhibit effective immune cell activation. In addition, the development of these new therapies will require designing clinical trials that efficiently assess combinations of biologics that target multiple immune pathways and incorporate validated predictors of immune response. Finally, demonstrating the success of these new therapies will likely require establishing new criteria to evaluate clinical responses that are associated with immune-mediated mechanisms of tumor control.

Keywords
Pancreatic cancer · Immunotherapy · Vaccine · Immune checkpoint · CTLA-4 · PD-1 · PD-L1 · TGF-β · IDO

Tumor Immunology and Pancreatic Cancer

Tumor Antigen Recognition and Immune Surveillance

The concept of cancer immune surveillance has been formulated based on the hypothesis that cancer cells are recognized as "non-self" and capable of inducing a rejection reaction. Cancer cells, although deriving from their normal counterparts, are distinguished by the expression of mutated, truncated, misfolded, improperly modified, overexpressed, aberrantly localized, or embryonic proteins. Autoantibodies against these proteins are detected in some cancer patients. These antibody-targeted proteins are considered to be tumor-associated antigens (TAAs). There is now ample evidence to demonstrate that spontaneous humoral and cellular immune responses can be detected in many cancer patients including those with pancreatic cancer.

Accumulating evidence from animal models provides strong support for the concept that tumor cells are recognized by host immune surveillance mechanisms (Fig. 1). Tumor cells expressing MHC class I genes can present tumor antigens directly to the predominant "killer cell" that mediates the rejection of tumor cells. These so-called $CD8^+$ or cytotoxic T lymphocyte (CTL) cells express clonotypically unique T cell antigen receptors (TCRs) that specifically recognizes a particular tumor antigen bound within the cleft of a major histocompatibility complex (MHC) class I molecule (human leukocyte antigen (HLA) type 1 in human). The recognition of

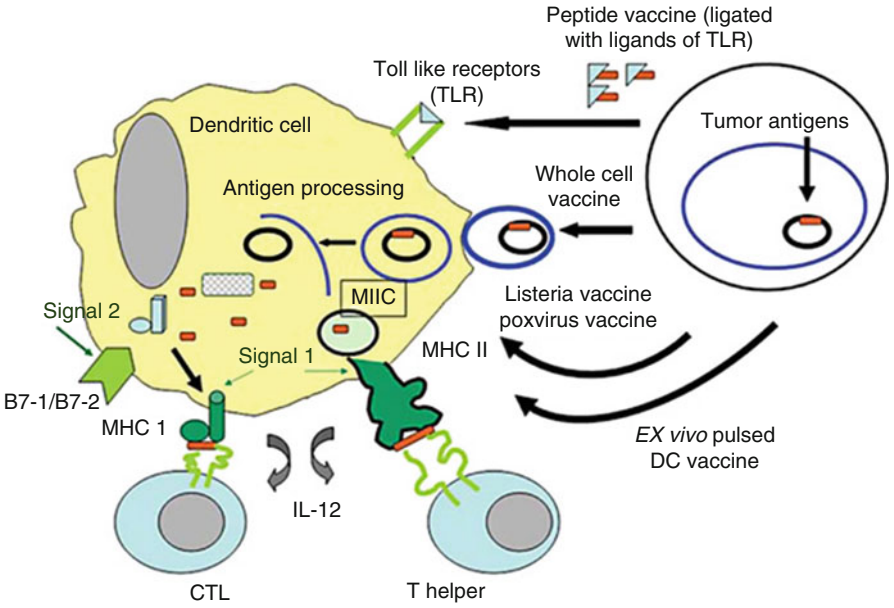

Fig. 1 APCs provide cross-presentation of tumor antigens and signals 1 and 2 for effective T cell activation. Dendritic cells (DC) are the most efficient APCs. These cells take up exogenous antigen from antigen-delivering vaccines [peptide, whole tumor cell, bacterial vector (*Listeria monocytogenes*), and viral vector (Pox virus vaccines)] and process these antigens on MHC class I and II molecules. Each vaccine has specific mechanisms for antigen entry. Some vaccines provide maturation signals to the DC by stimulating through Toll-like receptors (adjuvants, bacterial and viral vectors can do this). Activation of the DC results in enhanced presentation of antigen on MHC II and cross-presentation of antigen onto MHC class I molecules (signal 1) and the upregulation of signal 2 (B7-1/B7-2). Activation of DC also results in the production of pro-inflammatory cytokines such as IL-12 which further propagate T cell responses

antigen by TCR provides *signal 1* that is essential for T cell activation. Antigen recognition depends on the expression of MHC class I molecules on tumor cells. However, this signal alone is not enough to effectively activate a T cell. It is now well known that T cell activation also requires the binding of one or more co-stimulatory signals to its receptors on T cells, thereby providing what is termed *signal 2* [1]. However, most tumor cells are epithelial derived and therefore naturally lack expression of these important co-stimulatory signals. Instead, the successful activation of a T cell to recognize and lyse a tumor requires activation of professional antigen-presenting cells (APC), usually dendritic cells (DCs). DCs play a dominant role in processing and presentation. These cells have specific machinery for efficient uptake and processing of antigen onto their MHC molecules. They process exogenous antigens onto MHC class II for $CD4^+$ T cell activation and through, cross-presentation, onto MHC class I for $CD8^+$ T cell activation.

Historically, most cancers including pancreatic adenocarcinoma have been regarded as poorly immunogenic. More recent studies, however, have confirmed

the existence of tumor-reactive T cells and antibodies in the majority of cancer patients. For instance, a recent study reported that all pancreatic cancer patients investigated had high frequencies of tumor-reactive T lymphocytes in their bone marrow [2]. This concept is further supported by experimental evidence showing that mice with a variety of immunodeficiencies are more susceptible to carcinogen-induced and spontaneous tumors [3]. The central roles of immune effector cells such as B cells, T cells, natural killer (NK) cells, natural killer T cells (NKT), γδ-T cells, as well as type I and II interferons (INFs) and perforin, have long been proposed as cellular and cytokine mediators of cancer immune surveillance [4].

Immunoediting and "The Immunology of Carcinogenesis"

Cancers develop in patients who have a functional immune system with the potential to maintain surveillance against malignant cells. The model of cancer immunoediting has been proposed to delineate the carcinogenesis process as a dynamic equilibrium and disequilibrium process of immune surveillance and tumor escape [4]. The first step of immunoediting is the elimination phase. In this phase, immune surveillance successfully eradicates the developing tumor cells. This is started with the recognition of transforming cells by innate immune cells such as NK, NKT, γδ-T, and DCs. Following the maturation and migration of DCs to lymph nodes, these cells activate T cells, the adaptive component of the immune response. Tumor antigen-specific $CD4^+$ and $CD8^+$ T cells home to the site where the tumorigenesis process is initiating and CTLs eliminate the antigen-expressing cells that are undergoing transformation.

The second step is the equilibrium phase. During this phase, immune surveillance continues to eliminate tumor cells, while resistant tumor cells are selected out under pressure from the same elimination process. The random mutations in the genome of cancer cells may make them more susceptible to elimination. However, if the cancer cells acquire the wrong mutations that result in alterations in the expression of tumor antigens, the elimination process targeting these cancer cells would be weakened. Genetic instability and epigenetic alterations, which accompany the tumorigenesis process from normal cells to their malignant variants, provide opportunities for malignant cells to become less immunogenic cancer variants. The equilibrium phase may continue as long as abnormal cells derived from the tumorigenesis process can be eliminated by immune surveillance. Nonetheless, this equilibrium may eventually be disrupted and the immunoediting process reaches its final step, the escape phase. In this phase, cancer variants acquire genetic or epigenetic alterations making them insensitive to immunologic detection and elimination and are selected out. As a result, these transforming cells acquire dysregulated growth potential. Loss of tumor antigen expression is probably the most straightforward way for cancer cells to evade immunologic detection. Cancer cells always need to maintain some "non-self" features to distinguish them from their normal counterparts. However, cancer cells are able to sculpture the host immune system, for

example, by taking advantage of immune checkpoints (see below), and establish immune tolerance to these "on-self" features.

Mechanisms of Immune Tolerance

Alteration in T Cell Signal Transduction and Cytokine Regulation

Inflammatory signals are now recognized as contributors to the development and progression of most cancers. Cytokines, in particular, are often dysregulated during the process of tumorigenesis (Table 1). Pro-cancer cytokines are produced by many cells within the tumor's microenvironment including: stromal cells, APCs, regulatory T cells, endothelial cells, and the tumor cells themselves. These cytokines often downregulate activated cancer-targeted T cells, the mediators of antitumor immunity. Examples of two well-studied cytokines, TGF-β and IL-10, are described in detail below.

TGF-β

TGF-βs are regulatory molecules that affect multiple biological processes, including carcinogenesis and immune homeostasis via binding to its receptor, TGF-βR. TGF-βR is a heterodimer formed by TGF-βRI and TGF-βRII. TGF-b is a negative growth regulator. Binding of TGF-β to TGF-βRI activates TGF-βRII, which phosphorylates Smad2 and Smad3 and leads to their translocation into nuclei in a complex with SMAD4/DPC4 [5]. It is conceived that this nuclear translocation process allows Smad4/Dpc4 to function as a DNA-binding transcription factor in regulating genes involving cell growth, migration, and metastasis. Both SMAD4/DPC4 and TGF-β are thought to be tumor suppressors. Downregulation of

Table 1 Summary of mechanisms of immune tolerance

Mechanisms of immune tolerance	Regulatory components of tolerance
Alteration in T cell signal transduction and cytokine regulation	Upregulation of TGF-β signaling and IL-10
	Downregulation of IL-12 and IFN-γ
Tolerance induced by regulatory DCs and regulatory signals of DC differentiation	Immature DCs
	Upregulation of VGEF, COX-2, IL-6, and MCSF
	Downregulation of GM-CSF, IL-4, IL-12, and IFN-γ
Downregulation of co-stimulatory signals	Downregulation of B7-1 and B7-2
Immune checkpoints at the molecular level	Presence and/or upregulation of CTLA-4, PD-L1/B7-H1, PD-L2/B7-DC, B7-H3, B7-H4, PD-1
Cellular checkpoints of immune activity	Regulatory T cells (Tregs)
	Myeloid-derived suppressor cells (MDSC)
	Tumor-associated microphage (TAM)
Altered metabolism in immune cells	Upregulation of IDO, arginase and nitric oxide synthase, etc.

SMAD4/DPC4 mainly through loss of heterozygosity is found in 50–70% of advanced pancreatic cancers [6]. In the absence of a direct downregulation of SMAD4/DPC4, abnormal TGF-β signaling would still lead to the suppression of the function of SMAD4/DPC4. Reciprocally, downregulation of SMAD4/DPC4 renders tumor cells resistant to TGF-β-induced growth inhibition. Although TGF-β signaling suppresses tumor cell proliferation, it also plays an important role in negatively regulating immune cell function, rendering T cells tolerant to tumor growth. For example, genetic mice that are deficient in TGF-β or its receptor develop lethal autoimmune disease or severe inflammatory disease [5]. Furthermore, mice surviving to adulthood are resistant to challenge with tumors such as thymoma and melanoma. These studies provide evidence that T cells are direct targets of TGF-β and TGF-β regulates T cell responses specific for tumors.

TGF-β regulates a number of T cell populations and, in doing so, facilitates tumor growth and progression [5]. TGF-β suppresses $CD8^+$ T cells through multiple signaling pathways. It was suggested that TGF-β can suppress the expression of perforin, which is a key mediator of $CD8^+$ T cell killing of its target cells. In addition, suppression of IFN-γ production by $CD8^+$ T cells is thought to be mediated by Smad2 and Smad3, both of which are recruited to the promoter of INF-γ upon TGF-β-treatment. TGF-β regulation of $CD4^+$ T cells is less well understood. TGF-β potentially inhibits differentiation of Th1 and Th2 cells by inhibiting their lineage specification transcription factors such as T-bet and GATA-3. In addition, TGF-β induces expression of another transcription factor, FoxP3, which is a marker of $CD4^+$ $CD25^+$ Tregs. TGF-β is an important regulator of the homeostasis of Tregs (see below). In addition, TGF-β is a potent inhibitor of IL-12-induced production of IFN-γ in NK cells, suggesting its regulatory role in NK cell functions. Taken together, TGF-β represents an important mechanism of immune tolerance to tumors [5].

IL-10

Interleukin-10 (IL-10) is another important cytokine that mediates immune tolerance to tumors. IL-10 was initially identified as a molecule produced by Th2 cells and that inhibit productions of Th1 cytokines [7]. Ample evidence suggests that IL-10 blocks Th1 cell differentiation and proliferation and inhibits monocyte differentiation into DCs. In addition, IL-10-treated DCs fail to stimulate the cytotoxic activity of $CD8^+$ T cells. In addition, there is also evidence that IL-10 has direct effects on tumor cells to inhibit antitumor immune responses. For example, in human cancers, increased IL-10 expression and increased IL-10-producing immune cells are detected. IL-10 also downregulates HLA class I expression on tumor cells, thereby facilitating tumor escape from recognition by T cells. In one mouse model, transgenic expression of IL-10 results in a higher growth rate of an immunogenic lung carcinoma; whereas anti-IL-10 antibody or anti-IL-10 receptor antibody results in enhanced immune response to that same tumor. IL-10 is a pleiotropic molecule that displays both immunostimulatory and immunoregulatory activities. It has been shown to promote antitumor immune

responses in other mouse tumor models. Conceivably, the dual effect of IL-10 may originate from the differential roles of IL-10 on different tumor types. Therefore, it remains to be established how IL-10 regulates immune responses in two opposite directions [7].

Tolerance Induced by Regulatory DCs

A number of APCs are involved in the induction and maintenance of antitumor immune responses including DCs, monocytes/macrophages, and B lymphocytes [1]. DCs are the most potent among these APCs. As described above, to induce tumor immunity, sufficient numbers of functional APCs must present in situ, be able to capture, process, and present tumor-associated antigen, and subsequently stimulate TAA-specific T cells. Accumulating evidence has revealed that DCs have both a T cell activating and regulatory role in the induction and maintenance of antitumor immune response. Which role these cells play will depend on the initial signals provided within the context of the inflammatory response to the tumor.

The function of regulatory DCs can be characterized by the maturation state of these cells, specifically the surface molecules that they express. Matured DCs express high levels of surface markers such as CD40, CD80 (B7-1), CD83, and CD86 (B7-2) and produce high levels of IL-12 [8]. Mature DCs are functional and capable of inducing potent TAA-specific T cell immunity. Immature or partially differentiated myeloid DCs induce either suppressive T cells or T cell unresponsiveness. The interaction between the tumor environment and DCs provides another mechanism of tumor evasion [8]. Myeloid DCs arise from the same progenitor cells that also give rise to monocytes and macrophages [1]. However, the presence of functional immunogenic mature DCs is rare in human tumors. Many factors in the tumor environment may be responsible for the suppression of DC differentiation and maturation. Examples of these factors include VEGF, IL-6, MCSF, and COX-2 [8]. VEGF and COX-2 have been shown to suppress DC differentiation and maturation. IL-6 and macrophage colony-stimulating factor (MCSF) have been shown to switch DC differentiation toward macrophage differentiation. In addition, tumor cells, tumor-associated macrophages, and regulatory T cells produce IL-10 and TGF-β, which also suppress DC maturation and function. On the contrary, DC differentiation cytokines, such as GM-CSF and IL-4, as well as the Th1-type cytokines IL-12 and IFN-γ, are decreased in the tumor environment [8].

Downregulation of Co-stimulatory Signals

It is clear that the signals generated solely by TCR recognition of antigens are insufficient to activate T cells to an effector state. In fact, when T cells receive the only signal 1 through TCR engagement without additional co-stimulatory signals, they enter an unresponsive or anergic state. Signal 2, which is required for T cell activation, can be delivered by a number of co-stimulatory molecules [9]. The prototype of co-stimulatory molecules is B7-1 (CD80) and its homologue B7-2 (CD86). B7-1 and B7-2 co-stimulate T cells by interacting with the CD28 receptor on T cells. Unfortunately, co-stimulatory molecules are rarely expressed by tumor cells, representing another mechanism for the establishment of immune tolerance at

the local tumor site [9]. Therefore, the most successful vaccine approaches would be expected to stimulate immune responses through transfer of antigen to DCs, which naturally provide the necessary co-stimulatory signals when they present tumor antigenic peptide on MHC molecules to the T cell via TCR recognition.

Immune Checkpoints at the Molecular Level

CTLA-4

The positive regulatory effects of co-stimulatory signals are balanced by the presence of a number of co-inhibitory molecules. Although the binding of B7-1 and B7-2 to their CD28 receptor on T cells provides co-stimulatory signals, they can act as co-inhibitors when they bind to the cytotoxic T lymphocyte antigen 4 (CTLA-4) on T cells. The latter provides a co-inhibitory signal and decreases T cell activation both by outcompeting CD28 for ligand binding and inhibiting the signaling cascade that would be activated through the B7-1/B7-2-CD28 axis. CTLA-4 binds B7-1 and B7-2 with roughly 20-fold higher affinity than CD28 [10]. When naïve T cells are presented with antigen on B7-1 and B7-2-expressing APCs, they are co-stimulated because resting T cells express CD28 but not CTLA-4. Upon activation, CTLA-4 is expressed on T cells, thereby placing a "break" on the immune activation process (Fig. 2). The maintenance of the balance between stimulatory and inhibitory signals provides a mechanism to dampen unwanted responses once foreign antigens (infectious proteins) are cleared and ensures the tolerance to self-antigens and prevents autoimmune diseases. Knockout of CTLA-4 in mice confirms the importance of this signaling pathway since these mice succumb to lethal autoimmunity [11].

The discovery of this immune regulatory mechanism establishes the concept of immune checkpoints. CTLA-4 is the prototype of the molecules that govern immune regulation. It is likely that immunologic checkpoints serve two biological purposes. One helps generate and maintain self-tolerance among T cells specific for self-antigens. The other restrains the amplitude of normal T cell responses so that they do not "overshoot" in their natural response to foreign pathogens. The same immunologic checkpoint also gives tumor cells a chance at immune evasion. During tumor development, however, the balance leans toward co-inhibitory signals; and the presence of checkpoints plays a crucial role in the establishment of immune tolerance to tumors.

As a single intervention, anti-CTLA-4 monoclonal antibodies (mAb) can induce $CD8^+$ T cell-dependent tumor regression in tumor-bearing mice [11]. The primary activity of CTLA-4 mAb seems to be the prevention of CTLA-4 binding with B7-1 (CD80) or B7-2 (CD86). Combining CTLA-4 blockade with GM-CSF-secreting vaccination produces a synergistic antitumor effect compared to either alone in the non-immunogenic B16 melanoma mouse model [11]. Similarly, treatment with anti-CTLA-4 mAbs synergized with vaccination against a prostate-specific antigen (PSA) to induce antitumor effects in a transgenic model of spontaneous prostate cancer (TRAMP mice) [11]. Synergy with tumor vaccines has also been documented with synthetic peptide and DC vaccines. Anti-CTLA-4 antibody,

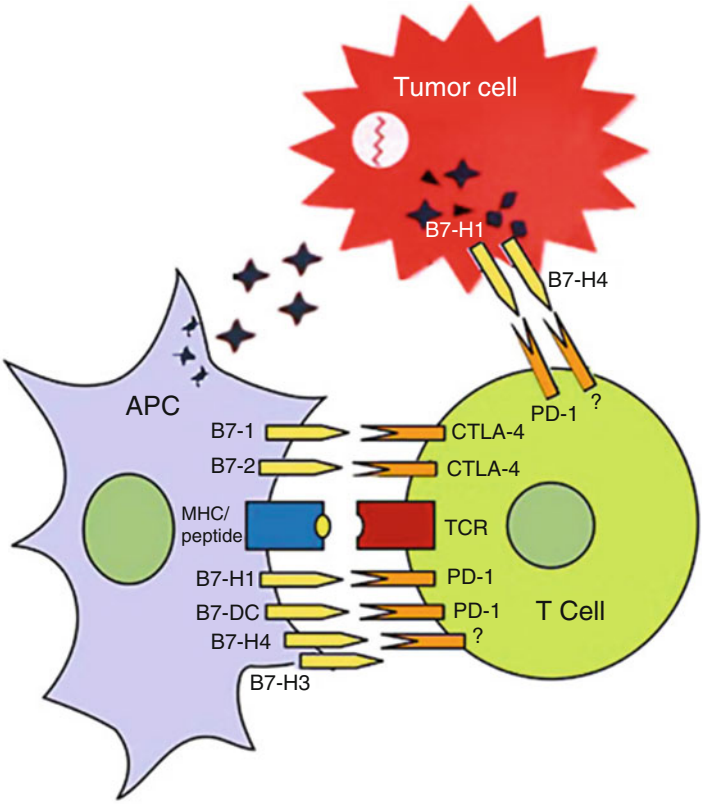

Fig. 2 Co-inhibitory signals and immune checkpoints. Tumor cells lack natural expression of co-stimulatory molecules such as B7-1/2. Therefore, T cells are not activated by tumor cells directly. When tumor cells provide signal 1 (HLA-peptide interacting with the TCR) without signal 2 (co-stimulation), T cell tolerance rather than activation results. Tumor cells may also express co-inhibitory molecules such as PD-L1/B7-H1 and B7-H4 and, by binding to their receptors on T cells, downregulate activated T cells. Tumor cells, tumor antigen peptide vaccines, or whole-cell vaccines can provide tumor antigens in a form appropriate for efficient processing and presentation by professional APCs, which then cross-present tumor antigens and have the potential to activate T cells. However, B7-1/B7-2 on APCs can also bind to the CTLA-4 receptor on T cells and deliver inhibitory signals that suppress T cell activation. Moreover, co-inhibitory molecules such as PD-L1/B7-H1 and PD-L2/B7-DC are present on APCs and can also provide inhibitory signals to T cells. These co-inhibitory molecules together with their ligands, CTLA-4 and PD-1 on T cells, respectively, constitute the immune checkpoints that are currently known to suppress antitumor immunity

ipilimumab, either alone or in combination with anti-PD-1 antibodies is now approved for treating advanced melanoma as described below in detail.

PD-L1, PD-L2, and Their Shared Receptor, PD-1

As discussed above, T cells harbor a natural co-inhibitory axis such as B7-1/B7-2-CTLA-4 that interacts with professional APC systemically (Fig. 2). In addition to

these systemic signals, T cells also express co-inhibitory signaling pathway that interacts with tumor cells and other cells within the tumor microenvironment. PD-L1 (B7-H1), another member of the B7 family, is an example of a co-inhibitory signal found on both DC and on many mouse and human tumor types [12]. Although resting T cells, B cells, and monocytes do not express PD-L1, they express high level of PD-L1 on their cell surface following activation. In contrast, DCs constitutively express PD-L1. Many types of tumor cells have been shown to have increased expression of PD-L1; and the tumor microenvironment can also stimulate the expression of PD-L1 on regulatory DCs [12]. In fact, both IL-10 and VEGF, two negative regulators of DC maturation and function, stimulate PD-L1 expression in myeloid DCs infiltrating human ovarian tumors and their draining lymph nodes [12]. PD-L1 also has a close homologue, PD-L2 (B7-DC), also in the B7 family. Expression of PD-L2 appears to be restricted to DCs and monocytes. PD-L2 also appears to be a co-inhibitory molecule [13]. Both PD-L1 and PD-L2 are ligands of PD-1, which is another inhibitory regulator expressed on the T cell surface (Fig. 2).

PD-1 shares significant homology with CD28, the receptor of co-stimulatory signals, B7-1 and B7-2 [14]. Its expression is induced upon activation of $CD4^+$ and $CD8^+$ T cells, B cells, and monocytes. PD-1 ligation to B7-H1/B7-DC causes inhibition of T cell activation and proliferation, which results in cell cycle arrest without apoptosis. The phenotype of PD-1 knockout mice is characterized by organ-specific autoimmunity [15]. PD-1 is particularly expressed by tumor-associated T cells, a significant fraction of which are Tregs [14]. Studies suggest that these tumor-associated, PD-1-expressing T cells can suppress antitumor immunity. So far, these PD-1-expressing tumor-associated T cells, through co-inhibitory signaling via PD-L1, have been shown to suppress IL-12 production by myeloid DCs, thus counteracting the positive effect of co-stimulatory signals. However, blocking PD-L1 has been shown to enhance myeloid DC-mediated T cell activation, allowing for suppression of growth of ovarian carcinoma xenografts following adoptive transfer of these cells into mice [12]. Administration of monoclonal antibodies (mAb) against PD-1 and B7-H1 has produced CTL-mediated antitumor effects in mice [14]. Therefore, tumor-associated PD-1 expression on T cells represents an additional mechanism of tumor evasion when B7-H1 is expressed by the progressing tumor. As described below in detail, anti-PD-1 and anti-PD-L1 antibodies have been approved for treating a number of malignant diseases.

B7-H3 and B7-H4

The B7 family is an expanding group of regulatory molecules expressed on professional APCs and some tumors. B7-1 and B7-2 were the first to be characterized followed by B7-H1 and B7-DC. B7-H4 is a more recently identified member of the B7 family [13]. Although B7-H4 protein expression is not as widely expressed as the other family members, aberrant expression has been demonstrated in human ovarian tumor-associated macrophages (TAMs) and human lung, breast, ovary, and renal cell carcinomas [13]. In addition, its expression can be upregulated in tumors following exposure to IFN-γ in vitro. Several lines of evidence support a role for B7-H4 in

mediating the immunosuppressive function of TAMs. B7-H4-positive TAMs are significantly more suppressive than B7-H4-negative TAMs. Blocking B7-H4 on TAMs disables their suppressive capacity. Furthermore, constitutive B7-H4 expression renders normal macrophages suppressive. B7-H4 has also been shown to interact with other tumor microenvironmental factors. For example, IL-6 and IL-10, which can be secreted by tumor cells, TAMs, or Tregs, stimulate monocyte/microphage B7-H4 expression. In contrast, GM-CSF and IL-4 reduce B7-H4 expression [16]. In addition, another B7 member, B7-H3, has also been proposed to be a co-inhibitor [12]. It is noteworthy that all these B7 molecules appear to have a dual function in immune regulation. Similar to B7-1 and B7-2, ligation of these B7 molecules can generate both positive and negative signals in T cells, depending on the context in which these T cells recognize their cognate antigen [12].

Regulatory T Cells Represent a Cellular Checkpoint of Immune Activity

Both mouse and human studies strongly support a major role for Tregs in mediating immune tolerance to tumors. These cells are characterized by high expression of $CD4^+$, $CD25^+$, and the FoxP3 promoter. These cells normally prevent autoimmune diseases by suppressing host immune responses when antigen load has been cleared. However, a growing number of reports have demonstrated that these cells are also recruited to tumor sites to inhibit antitumor immunity [17]. $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs normally comprise a small subset of the overall $CD4^+$ T cell population. However, their proportion is significantly elevated within the tumor microenvironment of many types of cancer. The cell surface molecule CD25 – encoding the IL-2 receptor protein – has been used as a marker for isolating Tregs. However, its expression is not restricted to Treg and is also detectable on many other activated lymphocytes including effector lymphocytes that mediate the antitumor immune response. The Forkhead box protein P3 referred to as FoxP3 has emerged as a highly specific marker of $CD4^+$ Tregs in both mice and humans [18]. Mutation of FoxP3 in mice and human causes a loss of Tregs and the production of an X-linked-recessive inflammatory disease and multisystem autoimmune syndrome. Furthermore, FoxP3 expression correlates well with suppressive activity of Tregs in both mice and humans.

While it is difficult to target a nuclear transcription factor like FoxP3, other Treg-selective cell and cell surface molecules have been identified. These molecules provide an opportunity to evaluate the selective targeting of Treg function (Fig. 2). One, designated glucocorticoid-induced tumor necrosis factor receptor (GITR), is a TNF receptor family member [19, 20]. Administration of anti-GITR antibodies enhances antitumor immunity in some murine systems, and it has been suggested that anti-GITR antibodies diminish the susceptibility of effector T cells to suppression by Tregs [21]. A second molecule, LAG-3, is a CD4 homologue that is selectively expressed on the surface of Tregs. Ectopic expression of LAG-3 confers suppressor activity upon $CD4^+$ T cells [22]. Blocking LAG-3 with a monoclonal antibody then inhibits the suppressive activity of Tregs. While surface expression of LAG-3 is very low on circulating Tregs, it is upregulated on Tregs in tissues and

tumors, suggesting that its role may be on activated Tregs at the site of immune suppression [23]. Although CTLA-4 is expressed on both effector T cells and Tregs, it plays an important role in suppressing effector T cells and mediating the suppressive function of Tregs. The exact role of CTLA-4 in conferring Treg function remains to be established because patients treated with anti-CTLA-4 antibodies do not show significant changes in the number or function of peripheral Tregs [24].

$CD4^+$ Tregs require antigen-specific activation or polyclonal TCR stimulation to exert their suppressive function. Once they are activated, they can suppress $CD4^+$ and $CD8^+$ T cells in an antigen-nonspecific manner. Several mechanisms have been proposed to explain how $CD4^+$ Tregs inhibit effector T cells. Most naturally occurring $CD4^+$ $CD25^+$ Tregs and antigen-specific Tregs both function through a cell-to-cell contact-dependent mechanism, while some antigen-induced Tregs can also suppress immune responses through soluble factors, including IL-10 and/or TGF-β-dependent mechanisms.

Tumors have been shown to induce rapid expansion of $CD4^+$ $CD25^+$ FoxP3 + Tregs in humans and mice, leading to delayed rejection of immunogenic tumors [25]. Conversely, elimination of these Tregs elicits potent antitumor immune responses leading to tumor eradication in mice [26]. Accumulated evidence has suggested the requirement of tumor-specific and pathogen-specific antigens for activating Tregs. The identity of these antigens remains largely unknown. These studies however support the existence of antigen-specific Tregs and the importance of tumor-infiltrating Tregs in suppressing antitumor immunity [27].

Other Immunosuppressive Cell Types

A distinct group of bone marrow-derived cells recently termed myeloid-derived suppressor cells (MDSC) are also directly involved in the suppression of immune responses to cancer. This cell population as well as aforementioned tumor-associated macrophages (TAM), respectively, may represent two other cellular checkpoints of immune activity. MDSC express both myeloid lineage differentiation antigen Gr-1 (Ly6G and Ly6C) and α_M integrin CD11b and represent 20–30% of normal bone marrow cells, 2–4% of all nucleated splenocytes, and are practically absent in lymph nodes. Inoculation with tumor cells or the development of spontaneous tumors results in a marked systemic expansion of these cells; and consequently, these cells become easily detectable in lymph nodes or tumor sites [28]. MDSC may exert an immunosuppressive effect in both an antigen-specific and nonspecific manner. It seems that at the tumor site, the immunosuppressive activity of MDSC is antigen nonspecific and is primarily mediated by the production of nitric oxide (NO) in combination with high arginase activity. Dysregulation of L-arginine metabolism in immune cells at the tumor site is reviewed in the next section [28]. In one genetic and spontaneous pancreatic tumor model, the presence of MDSC at the tumor site strongly correlates with the lack of tumor-infiltrating effector T cells with a near mutual exclusion. More interestingly, infiltration of immunosuppressive cells including MDSC, TAM, and Tregs, together with the lack of effector T cells, occurs at the early premalignant stage in pancreatic tumor development [29].

Altered Metabolism in Immune Cells

A number of metabolic pathways have been found to be altered in immune cells and to be associated with cancer development. Interest in indoleamine-2,3 dioxygenase (IDO) has grown rapidly with the discovery that IDO activity is critical for generating tolerance to foreign antigens [30]. IDO is one of two enzymes that degrade the essential amino acid tryptophan in mammals by catalyzing the initial, rate-limiting step in the pathway that produces nicotinamide adenine dinucleotide (NAD). In cancer, IDO is overexpressed in both tumor cells and stromal immune cells. When overexpression of IDO results in reduced tryptophan levels, antigen-dependent T cell activation in the tumor microenvironment is impaired [30]. In addition to tryptophan metabolism, the metabolism of L-arginine in tumor cells and its microenvironment is also altered in association with tumor growth. Arginase and nitric oxide synthase, two enzymes involved in L-arginine metabolism, are both over expressed. Accumulating evidence has supported the role of these enzymes as negative regulators of immune response to tumors [31]. Therefore, these altered metabolic pathways are likely utilized by cancer cells to induce immune tolerance.

Tumor Microenvironment is the Site Where Immune Tolerance is Established

One may ask why only antitumor immunity is specifically affected when so many aspects of the immune system response are deregulated. Indeed, only a minority of patients develop cancers due to their inherited immune deficiency. The majority of cancer patients have a healthy immune system and respond to other antigen stimulus such as infectious agents normally. This highlights the importance of the tumor microenvironment and tumor-infiltrating immune cells in establishing immune tolerance specifically to the developing tumor.

The tumor's microenvironment is the place where tumor cells interact with both immune cells and tissue-specific stromal cells. Depending on the inflammatory milieu at the time, this interaction may either restrain the proliferation, survival, invasion, and metastasis of tumor cells or facilitate tumor development and progression. Obviously, the tumor's microenvironment is also the place where tumor cells induce innate immune responses and where tumor antigens are processed and presented by DCs. An effective immune response is achieved by the homing of effector T cells to this microenvironment. At the same time, antitumor immune responses are also facing a strong immunosuppressive network and immune checkpoints (Fig. 3). Such an immunosuppressive network and the involved immune checkpoints must also be activated in this tumor microenvironment. Thus, the complex interaction occurring within the tumor's microenvironment is a dynamic process that at times involves opposing activity by the immune components that promote immune response and thereby inhibit tumor growth, as well as by those that impede immune response and facilitate tumor growth.

Cancers develop in part because the forces promoting the antitumor immune components are outcompeted by the inhibitors of the antitumor response within the tumor's microenvironment. There is an imbalance of mature versus immature DCs,

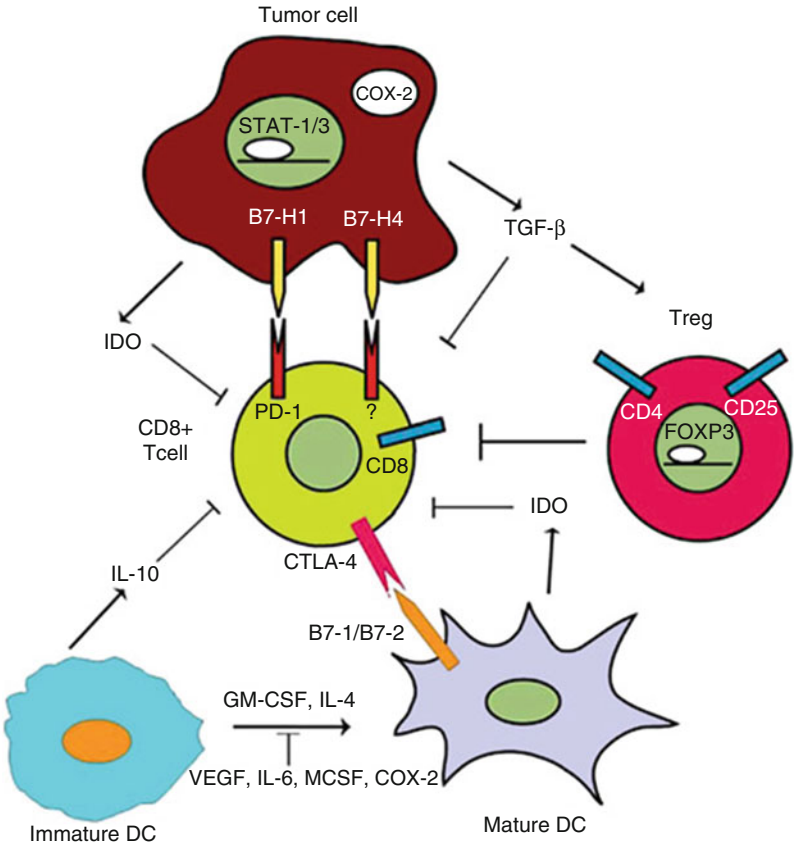

Fig. 3 An immunosuppressive network within the tumor microenvironment. The tumor's microenvironment is composed of a powerful immunosuppressive network, which includes immunosuppressive cells (e.g., Tregs), immunosuppressive cytokines (e.g., TGF-β, IL-6, MCSF, IL-10), and inhibitory signals provided by co-inhibitory molecules (e.g., PD-L1/B7-H1, PD-1, CTLA-4) on tumor cells, DCs, and T cells. In addition, immature DCs become predominant in the tumor microenvironment and contribute further to this immunosuppressive network. Moreover, tumor cells sculpt its microenvironment by secreting inhibitory molecules (e.g., upregulating IDO), rendering the microenvironment unfavorable for T cell activation

stimulatory versus inhibitory molecules, and effector T cells versus Tregs (Fig. 3). Increasing evidence suggests that immature DC is predominant in the tumor microenvironment, that co-inhibitory molecules such B7-H1 are highly expressed in tumor cells, and that Tregs are shown to infiltrate the tumor sites [8]. These imbalances start to occur at the earliest stages of tumor formation. One preclinical study has demonstrated that Tregs are already infiltrating the pancreatic in situ neoplasms (PanINs) that develop as a precursor to pancreatic tumors [29]. The author group has found that $FoxP3^{+}$ Tregs are infiltrating the majority of resected pancreatic tumors regardless of stage of these tumors (unpublished data). In addition to Tregs, two other

immunosuppressive cell types, MDSC and TAM, are also easily detected in the tumor sites and appear in the early-stage premalignant lesions during the development of mouse pancreatic tumors [29]. These studies strongly suggest that regulatory cells and molecules that are involved in immune checkpoint pathways are major components of the tumor microenvironment and likely from the earliest time of tumor initiation and development. In addition, tumor cells can sculpt their microenvironment and render it favorable for tumor escape. This is exemplified by the above described IDO pathway of immune cell regulation. By overexpressing IDO, tumor cells and their stromal cells create an environment that does not favor the proliferation and survival of effector T cells. Taken together, all evidence so far leads to the fact that the tumor microenvironment is the place where immune tolerance is established.

Limited by resources, tumor immunology research efforts have been focused on the systemic and peripheral immune responses and its associated regulation. To truly understand the immune tolerance mechanisms in the tumor microenvironment, however, it has become clear that the future delineation of these downregulatory pathways will require repetitive sampling of the tumor microenvironment. Understanding this dynamic process will lead to future therapeutic strategies that aim to re-sculpt the tumor microenvironment and render it favorable for antitumor immunity.

Immunotherapy Strategies

Passive Immunotherapy

Monoclonal Antibodies

The development of hybridoma technology has allowed the rapid production of monoclonal antibodies (mAbs) to target a single epitope. Since then, there has been a significant emphasis on the development of monoclonal antibodies that target tumor antigens and initiate tumor lysis either through direct signaling or through the delivery of a toxin conjugated to the monoclonal antibody. Advances in recombinant DNA technology allow the production of chimeric antibodies that contain the variable, antigen-specific region of the murine antibody and the constant regions of human antibodies. A further technology development is now allowing the production of fully humanized antibodies when mice genetically engineered with the human immunoglobulin gene is immunized with a human antigen. The first mAb approved for the treatment of cancer is rituximab (Rituxan), a chimeric anti-human CD20 mAb. Since its approval in 1997, many other mAbs have been approved for the treatment of cancer, and hundreds are undergoing preclinical and clinical evaluation [32] (the first nine approved mAbs listed in Table 2).

Rituximab is a chimeric anti-CD20 mAb that binds to human B lymphocytes and has been approved for treating B cell malignancies such as non-Hodgkin's lymphoma (NHL) either as monotherapy or in combination with chemotherapy. Ibritumomab tiuxetan (Zevalin) and tositumomab (Bexxar) are two radioimmunoconjugates directed against CD20. Trastuzumab (Herceptin) is a humanized

Table 2 Monoclonal antibodies with their approved indications

Generic name	Trade name	Antigenic target	mAb type	Approved indications
Rituximab	Rituxan	Anti-CD20 mAb	Chimeric	B cell malignancies
Trastuzumab	Herceptin	Anti-Her-2 mAb	Humanized	Her-2 expressing breast cancer
Alemtuzumab	Campath	Anti-CD52 mAb	Humanized	Chronic lymphocytic leukemia
Cetuximab	Erbitux	Anti-EGFR mAb	Chimeric	Metastatic colon cancer, squamous cell carcinoma of head and neck
Bevacizumab	Avastin	Anti-VEGF mAb	Humanized	Metastatic colon cancer, stage IV non-squamous NSCLC, metastatic breast cancer
Panitumumab	Vectibix	Anti-EGFR mAb	Humanized	Metastatic colon cancer
Gemtuzumab	Mylotarg	Anti-CD33 mAb	Humanized	Acute myeloid leukemia
Ibritumomab	Zevalin	Anti-CD20 mAb	Radioimmunoconjugated	B cell malignancies
Tositumomab	Bexxar	Anti-CD20 mAb	Radioimmunoconjugated	B cell malignancies

B cell malignancies include non-Hodgkin's lymphoma
mAb monoclonal antibody, *NSCLC* non-small cell lung cancer

mAb designed to bind to the extracellular domain of the human Her-2/neu receptor, a member of epidermal growth factor receptor (EGFR) family. It has been approved since 1998 for the treatment of metastatic Her-2-overexpressing breast cancer and was recently approved for the adjuvant treatment of Her-2-overexpressing breast cancer. Alemtuzumab (Campath-1H) is a humanized anti-CD52 mAb that is approved for the treatment of drug-resistant chronic lymphocytic leukemia (CLL). Cetuximab (Erbitux) is a chimeric mAb directed against Her-1, a member of EGFR family. Cetuximab was initially approved for the treatment of metastatic colorectal carcinoma and is now also indicated for the treatment of patients with squamous cell carcinoma of the head and neck. Bevacizumab (Avastin) is a humanized anti-angiogenic mAb that targets the vascular endothelial growth factor (VEGF). Bevacizumab is indicated in combination with 5-fluorouracil as first-line treatment

for metastatic colorectal cancer, advanced or metastatic non-squamous, non-small cell lung cancer, and metastatic breast cancer. Panitumumab (Vectibix) is a humanized mAb directed against EGFR and is approved for the treatment of metastatic colorectal cancer. Gemtuzumab ozogamicin (Mylotarg) is a humanized anti-CD33 mAb conjugated to calicheamicin, a cytotoxic antibiotic. CD33 is a glycoprotein receptor expressed on normal and monomyeloid hematopoietic progenitor cells. Gemtuzumab ozogamicin is approved for the treatment of patients with acute myeloid leukemia (AML).

The in vivo antitumor mechanisms of these monoclonal antibodies are proposed, including specific blockade of the function of proteins that they are directed against, antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), growth inhibition, apoptosis, and chemo- and radiosensitization of tumor cells, and enhanced antigen processing and prevention by DC [32]. Many more mAbs are being evaluated in preclinical and clinical studies, including those for the treatment of pancreatic cancer. These studies in the following sections will be discussed below.

Adoptive T Cell Transfer

T cells are considered the most powerful tools of the adaptive immune response that are capable of specifically recognizing and lysing tumors including pancreatic cancers. Adoptive transfer of T cells has been tested in preclinical and clinical studies [33]. In an early study, mice bearing disseminated leukemia were successfully treated by a combination of cyclophosphamide and adoptive transfer of syngeneic immune lymphocytes. Another early study showed that an injection of spleen cells activated in vitro by IL-2 resulted in tumor rejection. The antitumor effect of adoptive T cell transfer has been further supported by clinical studies showing that adoptive transfer of highly selected tumor-reactive T cells could mediate tumor regression. However, a number of obstacles remain to be overcome before adoptive T cell transfer can be applied routinely for the treatment of many patients with cancer [33]. These obstacles include overcoming the feasibility of routinely expanding to therapeutic numbers of an activated population of T cells specific for the tumor and maintaining a prolonged activated state in patients following adoptive transfer. More recent studies would suggest that successful treatment of cancer with adoptively transferred T cells requires a pre-conditioning chemotherapy regimen that inhibits mechanisms of in vivo T cell downregulation [34].

Immunesuppressive cells such as Tregs are probably the main mechanistic obstacle to overcome for adoptive T cell transfer to achieve potent antitumor activity in vivo. Others include the competition between endogenous T cells and adoptively transferred T cells for cytokines important for their growth and activation, and the difficulty for transferred cells to traffic to the tumor site and into the tumor. To overcome these obstacles, lymphodepletion to remove endogenous T cells and Tregs was tested in combination with adoptive T cell transfer. Other solutions have been proposed to enhance migration of T cells toward tumors including transducing transferred T cells with chemokine receptors. As retroviral gene transfer involves

extensive in vitro stimulation, which triggers T cell differentiation but also loss of expression of certain chemokine receptors and adhesion molecules, new protocols have been proposed to avoid in vitro T cell activation [33].

From a feasibility point of view, the requirement of ex vivo culture and sophisticate techniques is a major limitation for the translation of adoptive T cell transfer into routine oncology practice. Moreover, it is difficult for any single laboratory to isolate antigen-specific T lymphocytes reproducibly. Chimeric antibody receptor T cell therapy overcomes this challenge by providing antigen specificity through genetic engineered antibody receptors [35]. This technology has demonstrated its success in treating lymphoblastic malignancies and showed its promising efficacy in other hematologic malignancies. However, CAR T therapy is associated with cytokine release syndrome, which could become fatal and thus limit the application of CAR T therapy [36]. There are also enthusiasms of developing CAR T therapies for solid malignancies including pancreatic cancer [36]. Nevertheless, CAR T therapies in solid tumors face additional challenges including the difficulty for CAR T cells trafficking into tumors.

Cloned T cell receptor (TCR) genes can be used to produce T lymphocyte populations of desired specificity and offers new opportunities for antigen-specific T cell therapy. Several groups have demonstrated the feasibility of retroviral TCR gene transfer to produce antigen-specific TCR expressing T cell populations that function in vivo [33]. Recently, human mesothelin-specific TCRs have been cloned; and mouse mesothelin-specific TCR-cloned T cells were found to be able to enter the pancreatic tumors that are spontaneously developed in transgenic mice and highly resemble human pancreatic ductal adenocarcinoma [37].

Sipuleucel-T (Provenge) is one of few DC-based vaccines that have been tested in late phases of human studies [38]. Sipuleucel-T is made with mature, autologous DCs obtained from the patient via a standard leukapheresis. The antigen-loaded DCs are reinfused to the same patient in 3–4 days following the initial leukapheresis. Two phase III studies for the treatment of metastatic hormone-refractory prostate cancer patients have been completed. Although the primary endpoint, time to disease progression was not met, overall survival was improved with a statistical significance in patients treated with Sipuleucel-T compared with those treated with placebo [38]. Sipuleucel-T eventually became the first FDA-approved cancer vaccine and is indicated for metastatic hormone-refractory prostate cancer [39]. Like Sipuleucel-T, other DC-based immunotherapies demonstrate their safety in the clinical trials and showed their promising efficacy results. Because DC-based vaccines are different from conventional drugs and require multiple procedures with sophisticate technologies, the feasibility of such an immunotherapy modality being a routine cancer treatment has remained a challenge. Another major concern with the current form of DC vaccines is the lack of studies that have evaluated the best routes of administration and the best preparations for optimal immunization of large numbers of patients. Unfortunately, current methods have significant patient-to-patient variability within a given study and between different studies.

Targeting CTLA-4

As the major obstacles for effective antitumor immunity are immunologic checkpoints, the cells (Tregs) and molecules that convey checkpoint signals have become the targets for immunotherapy. Many agents have been or are being developed to target immunologic checkpoints. Among them, monoclonal antibodies specific for CTLA-4 was the first of the class tested in preclinical models and human studies. The studies of monoclonal antibodies against mouse CTLA-4 have been reviewed above.

Human CTLA-4 monoclonal antibodies have also been developed. Ipilimumab and tremelimumab, two different humanized monoclonal antibodies directed to human CTLA-4, have been independently tested in multiple clinical studies including phase III clinical trials in advanced melanoma [40, 41]. These trials have led to the FDA approval of ipilimumab for melanoma. However, grade III/IV autoimmune toxicity is also highly notable and can in some cases result in death if not treated quickly and effectively. In particular, colitis has been observed in approximately 30% of patients treated, often requiring steroid intervention for alleviation of symptoms [40, 41].

Targeting PD-1

PD-1 blockade monoclonal antibodies in the class of checkpoint inhibitors are subsequently approved by the US Food and Drug Administration (FDA). Pembrolizumab and nivolumab are both humanized monoclonal antibody directed again human PD-1.

Nivolumab has been approved by FDA to treat advance melanoma as the first-line therapy, metastatic non-small cell lung cancer (NSCLC) as a second-line treatment, renal cell carcinoma as the second-line therapy, metastatic or recurrent squamous cell carcinoma of the head and neck as the second-line therapy, and recurrent Hodgkin's lymphoma. Pembrolizumab has been approved by FDA to treat advanced melanoma and PD-L1-positive metastatic NSCLC as the first- and as the second-line therapies [42–52]. No dose-limiting toxicity has been observed in the phase I studies of either antibody.

These antibodies have a lower toxicity profile than anti-CTLA antibody [42, 43]. This is anticipated on basis of the result of PD-1 knockout mice developing mild strain-dependent, organ-specific autoimmunity, in contract to CTLA-4 knockout mice that develop lethal multi-organ autoimmunity [15]. Second, it may have a relatively specific role in blocking T cell suppression in the tumor microenvironment. This assumption was made upon the evidence showing that PD-L1, the PD-1 ligand, is highly expressed in a variety of human tumors [53]. In contrast, the CTLA-4 ligands are systemically expressed on APCs. Similar to CTLA-4 blockade, preclinical models have shown that PD-1 blockade synergizes with tumor vaccines [54].

Targeting B7 Family and Other Checkpoint Molecules

Other co-inhibitory molecules in the immune checkpoints are also potential targets for therapy. Monoclonal antibodies specific for co-inhibitory molecules, PD-L1/B7-H1,

PD-L2/B7-DC, B7-H3, and B7-H4, have been tested in preclinical models and have all been shown to augment T cell immunity [13]. As described above, these ligands can also deliver costimulatory signals, presumably through different receptors. Therefore, it will be more challenging to employ therapeutics that targets these molecules. Extensive preclinical modeling of these checkpoint inhibitors should inform the early clinical trials as to the best way to employ the targeted agents. Nevertheless, anti-PD-L1 antibodies have been shown to have similar antitumor efficacies and safety profiles as anti-PD-1 antibodies in multiple cancer types [42, 43]. Moreover, atezolizumab, an anti-PD-L1 antibody, has been approved by FDA to treat the advanced urothelial carcinoma as the second-line therapy [55].

In addition, therapeutic agents that target other checkpoint molecules such LAG-3, TIM-3, etc. are also being tested in clinical trials as single agents or in combination with anti-PD-1/PD-L1 antibodies [56].

Targeting IDO

IDO is a molecule secreted within the tumor's microenvironment that also functions as an immune checkpoint through regulating T cell metabolism. A small number of studies have offered evidence that IDO inhibition with 1MT or other small-molecule inhibitors can exert antitumor effects [30]. Although 1MT by itself was unable to elicit tumor regression, the delivery of 1MT in combination with a variety of classical cytotoxic chemotherapeutic agents elicited regression of mammary tumors in HER-2/neu transgenic mice. Immunodepletion of CD4+ or CD8+ T cells from the mice before treatment abolished the combinatorial efficacy observed in this model, confirming the expectation that 1MT acted through activation of T cell-mediated antitumor immunity. In addition, small-molecule inhibitors of IDO including several thiohydantoin derivatives of tryptophan have been identified, and administration of these inhibitors resulted in the same pattern of antitumor properties as 1MT. IDO has a number of appealing pharmacodynamic features as a target for drug developments. Design and development of more efficient IDO inhibitors is underway. Phase I/II studies of IDO inhibitors for human cancers have shown promising results [57, 58].

Targeting Tregs

Tregs are the key cellular component of immune checkpoints. So far, none of the targeted strategies are able to specifically block or deplete Tregs in human cancer patients. Anti-CD25 antibodies have been proposed to deplete Tregs. However, in the setting of human vaccine trials, this strategy would likely be flawed because CD25 is expressed on both CD4+ CD25+ Tregs and newly activated effector T cells. Indeed, clinical trials using ONTAK (an IL-2 toxin fusion protein that binds CD25) show either inefficient elimination of Tregs or both depletion of Tregs and suppression of some important aspects of tumor-specific immune responses [59]. Also, CTLA-4 blockade does not completely overlap with Treg inhibition. Studies of cyclophosphamide, a chemotherapeutic that inhibits Treg populations when given in immune-modulating doses or in very high dose, are elucidating some of the

specific mechanisms for inhibiting Tregs. It cannot be overemphasized that therapeutic strategies targeting Tregs are in high demand.

Adoptive T Cell Transfer for Pancreatic Cancer Immunotherapy

Adoptive T cell immunotherapy has also been tested in advance pancreatic cancer. Twenty patients with unresectable or recurrent pancreatic cancer were treated by both dendritic cells pulsed with MUC1 peptide (MUC1-DC) and cytotoxic T lymphocytes (CTL) sensitized with a pancreatic cancer cell line expressing MUC1 (MUC1-CTL). Peripheral blood mononuclear cells (PBMCs) obtained from an individual patient were separated into adherent cells for induction of MUC1-DCs and floating cells for MUC1-CTLs. Following ex vivo activation, MUC1-DC and MUC1-CTL were transferred back to the patient. Patients were treated from 2 to 15 times. One patient with multiple lung metastases experienced a complete response. Five patients had stable disease. The mean survival time was 9.8 months. Only grade I toxicity was observed. It would be difficult to distinguish between the effects of DC therapy and adoptive T cell transfer in this study. Nonetheless, this study suggested that adoptive immunotherapy with MUC1-DC and MUC1-CTL is safe and may be feasible for pancreatic cancer [60]. MUC1-specific and mesothelin-specific CAR T therapies are also being tested in the clinical trial [61, 62].

Development of Pancreatic Cancer Vaccines

Antigen-Specific Vaccines

Tumor Markers as Vaccine Antigens

The vaccine trials in pancreatic cancer were first designed to target a defined pancreatic cancer antigen. However, such an approach requires a comprehension of pancreatic cancer antigens that are immunogenic. For a long time, tumor markers have been an obvious option. The idea has been that molecules associated with pancreatic cancer and used for diagnostic purposes, e.g., CEA, MUC1, gastrin, etc. could also be used as therapeutic vaccines [63] (Table 3).

The high level of expression of both CEA and MUC1 by pancreatic cancers suggested that combining vaccination against both antigens might be appropriate in this disease. In one study, a phase I trial was conducted using an admixture of vaccinia virus expressing MUC1 with a vaccinia-CEA-TRICOM vaccine for priming followed by booster immunizations using fowlpox-CEA-TRICOM. This prime and boost regimen was based on preclinical studies showing that sequencing these pox viruses in this way enhanced antigen-specific immunity against the tumor while avoiding vaccinia-specific immunity that might mask cancer-specific immunity in patients who were previously vaccinated with vaccinia to prevent small pox disease [64]. Patients with metastatic or locally advanced pancreatic cancer who had failed prior chemotherapy were eligible. A second phase I trial was carried out using the

Table 3 Current progresses on human pancreatic cancer vaccine studies

Studies	Patient characters	Vaccine type and treatment	Immunologic analysis	Clinical efficacy
Phase I PANVAC-VF [65]	Eight evaluable patients, advanced pancreatic cancer, heavily pretreated	CEA-MUC1-TRICOM in poxvirus plus recombinant GM-CSF	Antigen-specific T cell responses in five out of eight patients; significant increase in OS in patients who generated anti CEA- and/or MUC1-specific immune responses compared with those who did not (15.1 vs. 3.9 mo, $P = 0.002$)	Median OS of 6.3 mo
Phase III PANVAC-VF (unpublished)	250 patients, metastatic pancreatic cancer	CEA-MUC1-TRICOM in poxvirus plus recombinant GM-CSF versus best supportive care	Not reported	No improvement in overall survival
Phase III gastrin peptide [66]	154 patients, advanced pancreatic cancer, unwilling or unsuitable to take chemo	Peptide vaccine (G17TD) versus placebo	Not reported	Median OS of 151 versus 83 d, $p = 0.03$
Phase III gastrin peptide [67]	Advanced pancreatic cancer	Gemcitabine + peptide vaccine (G17TD) versus gemcitabine + placebo	Not reported	OS (178 vs. 201 d) TTP (118 vs. 118 d) RR (21 vs. 23%)
Pilot study of mutant ras peptide [68]	5 patients	Peptide vaccine	2/5 showed immune response specific for individual ras mutations and also had a relatively longer survival. These two patients demonstrated vaccine-induced CD4+ and CD8+ T cell response specific for ras epitopes containing G12D mutation	2/5 had a relatively longer survival

Phase II study of mutant ras peptide [69]	48 patients: 10 surgically resected; 38 with advanced pancreatic cancer	Peptide vaccine plus recombinant GM-CSF	Peptide-specific immunity induced in 58% of patients. Patients with advanced cancer demonstrating an immune response to the peptide vaccine showed prolonged survival compared to nonresponders (median OS 148 vs. 61 d)	Median OS (responders vs. nonresponders): 148 versus 61 d
Phase II mutant ras peptide [70]	11 patients: 5 resected pancreatic cancer, 6 resected colon cancer	Peptide vaccine Adjuvant treatment	Specific immune responses to the relevant mutant ras peptide were detected in 5 out of 11 patients	A mean disease-free survival of 35.2 + months and a mean OS of 44.4 + months
Phase I/II GV1001 [71]	48 patients, non-resectable pancreatic cancer	Telomerase peptide vaccine plus GM-CSF	Immune responses measured as DTH and in vitro T cell proliferation were observed with the highest ratio in the intermediate dose group	Median OS for the intermediate dose group was 8.6 mo, significantly longer than the low- and high-dose groups
Phase III GV1001 [73]	1065 patients, locally advanced or metastatic pancreatic cancer	Chemotherapy (gemcitabine plus capecitabine) versus chemotherapy and sequential or simultaneous GV1001 vaccine	Not reported	Chemotherapy alone: 7.9 months; sequential chemoimmunotherapy: 6.9 months; concurrent chemoimmunotherapy: 8.4 months. not significantly different
Phase I/II personalized multiple peptide [75]	20 patients, metastatic pancreatic cancer	Multi-peptide vaccine	Vaccination-augmented peptide-specific T cell responses and IgG titer were observed in 72% and 78% of the patients, respectively	Median OS was 8.5 mo: 5 PR; 11 SD.

(*continued*)

Table 3 (continued)

Studies	Patient characters	Vaccine type and treatment	Immunologic analysis	Clinical efficacy
Phase I HSPPC-96 [76]	11 patients, advanced pancreatic cancer	Autologous tumor-derived gp96 heat-shock protein – peptide complex combinatorial treatment with gemcitabine	Autologous anti-HSPPC-96 ELISPOT reactivity increased significantly in only one of 5 patients examined	Three of 10 treated patients were alive without disease at 2.6, 2.7, and 5.0 years follow-up. Median OS was 2.2 years
Phase I allogeneic pancreatic cancer vaccine [77]	14 patients, resected adenocarcinoma of pancreas	GM-CSF secreting, allogeneic whole-cell vaccine, adjuvant treatment in sequence with chemoradiation	3/14 subjects developed DTH to autologous tumor cells	3/14 subjects experienced prolonged disease-free survival
Phase II allogeneic pancreatic cancer vaccine [78]	60 patients, resected pancreatic adenocarcinoma	GM-CSF secreting, allogeneic whole-cell vaccine, adjuvant treatment in sequence with chemoradiation	Correlation between disease-free survival and the induction of mesothelin-specific T cell responses	1 year survival, 86%; 2 year survival, 61%; median OS, 24.8 mo
Phase I/II allogeneic pancreatic cancer vaccine [80]	50 patients, advanced pancreatic cancer, $\geq$ 2 prior chemotherapy regimens	GM-CSF secreting, allogeneic whole-cell vaccine cohort A ($n = 30$), vaccine alone; cohort B ($n = 20$), Cy + vaccine	Detected the enhanced mesothelin-specific T cell responses in vaccinated patients	Clinical response: cohort A, 5 SD, 23 PD; cohort B, 6 SD, 11 PD; TTP & OS: cohort A, 1.4 & 2.3 mo; cohort B, 1.9 & 4.7 mo
Phase I MUC1-pulsed DC-based vaccine [74]	20 patients, advanced pancreatic cancer	DCs pulsed with MUC1 peptide plus CTL sensitized with MUC1-expressing pancreatic cancer cells	Not reported	One patient with multiple lung metastases experienced a complete response. Five patients had SD. Mean OS 9.8 mo

SD stable disease, *PR* partial response, *PD* progressive disease, *mo* month, *d* days, *wk.* weeks, *Cy* cyclophosphamide, *TTP* time to progression, *RR* response rate

PANVAC-VF regimen. This consists of priming with PANVAC-V (a single vaccinia vaccine co-expressing CEA, MUC1, and TRICOM) followed by three booster doses of PANVAC-F (fowlpox vaccines expressing the three transgene components). The vaccines were administered ever 2 weeks by subcutaneous injection followed by local recombinant GM-CSF adjuvant for 4 days. Monthly booster vaccinations for up to 12 months were provided for patients without progressive disease. Antigen-specific T cell responses were observed in 5 out of 8 evaluable patients (62.5%). Median overall survival was 6.3 months and a significant increase in overall survival was noted in patients who generated anti CEA- and/or MUC1-specific immune responses compared with those who did not (15.1 vs. 3.9 months, respectively; $P = 0.002$). Although the subject number is small in this study, a median overall survival of 6.3 months in advanced pancreatic cancer patients who had been heavily pretreated with chemotherapy appeared favorable and thus led to a phase III trial in advanced pancreatic cancer patients [65]. This randomized controlled phase III clinical trial enrolled 250 metastatic pancreatic cancer patients. Patients were randomized 1:1 to either vaccine or best supportive care. Patients randomized to the PANVAC-VF arm of the trial received 2×10^8 pfu of PANVAC-V followed by 100 μg GM-CSF. Subsequently, these patients receive 1×10^9 pfu PANVAC-F also followed by GM-CSF. Patients who did not have progressive disease received monthly booster immunizations. Unfortunately, this study did not meet their primary efficacy endpoint of improving overall survival [64]. There are many proposed reasons for the failure to show significant activity. The most likely reason is that this study provided a vaccine to patients with significant immune-tolerizing mechanisms, thereby precluding access and function of vaccine-induced T cells in this patient population.

Another large antigen-specific vaccine study tested a peptide vaccine, G17DT, that targets the antigen, gastrin. In this study, 154 pancreatic cancer patients unsuitable or unwilling to take chemotherapy were treated with either placebo or a gastrin peptide vaccine. Median survival was 151 days in the vaccine group versus 82 days in the placebo group ($p = 0.03$). In the previous phase II study, anti-gastrin antibody responders demonstrated significantly greater survival than antibody nonresponders. Immune response endpoints have not yet been reported for this phase III study [66]. Nonetheless, in a follow-up study in which patients with advanced pancreatic cancer were randomized to receive gemcitabine with or without G17DT, there was no major difference between gemcitabine plus vaccination with G17DT versus gemcitabine plus placebo for overall survival (178 days vs. 201 days), time to tumor progression (118 vs. 118 days), or response rate (21% vs. 23%) [67]. Thus, the approach of using tumor markers as vaccine antigens may or may not be effective, depending on the vaccine approach employed and on the patient population in which the vaccine is being tested. It is conceivable that immune tolerance to these antigens have been established long before carcinogenesis is initiated since these antigens are also expressed by the normal tissue from which the tumor derives. It would be unlikely to easily overcome tolerance to these self-antigens as such a tolerance is critical for the protection of the normal cells.

Oncoproteins as Vaccine Antigens

Another vaccine approach takes advantages of genetic and epigenetic changes that occur during the carcinogenesis process. This approach is attractive because it provides an opportunity for targeting tumor-specific antigens. Several pancreatic cancer-associated oncoproteins have been used as vaccine targets. As mentioned above, Kras mutations occur frequently in pancreatic cancers. In a pilot trial of a mutant Kras peptide vaccine, two out of five pancreatic cancer patients showed immune response specific for individual Kras mutations and also had a relatively longer survival. These two patients demonstrated vaccine-induced CD4+ and CD8+ T cell responses specific for Kras epitopes containing the substitution from glycine to valine at codon 12 [68]. In a second trial, mutant Kras peptide vaccine was given to 48 patients (10 surgically resected and 38 with advanced disease) together with GM-CSF as an adjuvant. Peptide-specific immunity was induced in 58% of evaluable patients. Patients with advanced cancer demonstrating an immune response to the peptide vaccine showed prolonged survival compared to nonresponders (median survival 148 days vs. 61 days, respectively) [69]. A follow-up phase II study of this vaccine as an adjuvant treatment in pancreatic cancer and colorectal cancer was also reported. Vaccinations were given every 4 weeks, up to a total of six vaccines. Specific immune responses to the relevant mutant ras peptide were detected in five out of 11 patients. Furthermore, the five pancreatic cancer patients have shown a mean disease-free survival of 35.2 + months and a mean overall survival of 44.4 + months [70]. Although the survival outcome appears positive, it is difficult to judge the exact benefit of the vaccine with such a small subject number in this study. None of these vaccine studies report serious adverse effects, however, suggesting that such an approach is safe and feasible and warrants further investigation particularly in combination with other therapies.

As telomerase is reactivated in most tumor cells, it has also become a target for peptide vaccines. In a dose escalation phase I/II study, 48 patients with newly diagnosed non-resectable pancreatic cancer were treated with peptide vaccines (GV1001) targeting the hTERT subunits of telomerase. GM-CSF was also used as an adjuvant. The vaccine was injected intradermally eight times over a period of 10 weeks followed by monthly booster vaccinations. The vaccine was tested at three dose levels and was well tolerated. Immune responses measured as delayed-type hypersensitivity (DTH) to the immunizing peptides and in vitro T cell proliferation were observed with the highest ratio in the intermediate dose group. Consistently, median survival for the intermediate dose group was 8.6 months, which was significantly longer than the low- and high-dose groups [71]. Thus, a prospective, phase III, controlled, multicenter, randomized clinical trial (TELOVAC) is comparing combination gemcitabine and capecitabine therapy with concurrent and sequential GV1001 treatment in locally advanced and metastatic pancreatic cancer was followed. The rationale for the combinatorial therapy with gemcitabine and vaccination is based on a report suggesting that patients vaccinated during the first week of chemotherapy following surgical resection mounted both cellular and humoral

responses to a standard panel of microbial antigens measured 12 weeks after vaccination [72]. A second report further suggested that T cells from patients undergoing gemcitabine treatment were functional and that gemcitabine may decrease memory T cells and promote naïve T cell activation. The phase III study of GV1001 was designed to test the hypothesis that combining cancer vaccines with standard gemcitabine treatment in patients with pancreatic cancer is feasible and may result in synergistic effects. This study was conducted at multiple centers by the Pancreatic Cancer Subgroup of the National Cancer Research Institute in the United Kingdom and randomized 1062 patients to receive either chemotherapy alone, chemotherapy with sequential GV1001, or chemotherapy with concurrent GV1001. Nevertheless, it failed to demonstrate the survival benefit of GV1001 in either sequential or concurrent combination with chemotherapy [73].

Although much still needs to be learned about the optimal antigens for pancreatic cancer vaccination, the ideal antigen will likely lack of pre-existing tolerance, be selectively expressed by the tumor, and indispensable for maintaining the malignant phenotype of the tumor cells. Oncoproteins are a category of antigens that may have all of these features. However, it has become apparent that even mutated tumor-associated antigens can be viewed as "self-antigen" by the body. As described above, the establishment of tolerance parallels the process of carcinogenesis. Moreover, carcinogenesis varies from one patient to another. None of the events are shared by all patients with a given tumor type including pancreatic cancer. Even for the same oncoprotein, different mutations may occur in different patients. Thus, it is almost impossible to employ the same oncoprotein peptide to vaccinate all patients even though the HLA type would not be considered as a variable factor. Finally, it would be wrong to assume that an oncoprotein plays a critical role in maintaining the malignant phenotype of the tumor cells at all times during tumor development and progression. With an instable genome, a tumor cell could easily acquire additional mutations which render the original oncogenic mutation dispensable for the maintenance of the malignant phenotype. It is not difficult to conceive that the resulted tumor variants, designated antigen loss variants, occur frequently under the pressure of immune surveillance. Therefore, it is unlikely that any single antigen would become an ideal vaccine target. Meanwhile, even an ideal vaccine needs to be combined with a treatment that can break the multiple mechanisms that contribute to immune tolerance.

DC-Based Vaccines

Several antigen pulsed DC vaccines have been designed for pancreatic cancer treatment. A phase I/II clinical trial of the vaccine composed of MUC1 peptide loaded DCs was tested in 12 patients with resected pancreatic cancer; however, most patients did not exhibit an overall increase in T cell functionality at the completion of the trial compared to pre-vaccine levels [74]. As mentioned earlier, DC vaccines have not been standardized between studies and between patients within a study. Therefore, the results of studies like these are difficult to assess due to interpatient variability as well as intrapatient variability of the administered treatments.

Mixed Antigens as Vaccine Targets

Personalized vaccines that target multiple tumor antigens have been explored as alternative approaches to overcome the need for knowing the best antigen or antigens to target in a given patient and to bypass the potential loss of expression of a single antigen. In one personalized vaccine approach, peripheral blood mononuclear cells and plasma were obtained from each individual patient to examine their cellular and humoral responses to 23–25 peptides prior to vaccination. Only the reactive peptides (maximum of four) were then administered to the patient. In a recent phase I/II study, gemcitabine was given intravenously once a week for three out of every 4 weeks, in sequence with the administration of three or four reactive peptides given once a week. Twenty patients with metastatic pancreatic adenocarcinoma were treated, resulting in a partial response in 5 patients and stable disease in 11 patients. The median overall survival was 8.5 months. Vaccination-augmented peptide-specific T cell responses and IgG titers were observed in 72% and 78% of the treated patients, respectively. Correlation between clinical responses and immune responses were not reported [75].

Another vaccine approach that potentially targets multiple tumor antigens is the heat-shock protein-peptide complexes. This vaccine approach takes advantage of the in vivo noncovalent binding between chaperone proteins and tumor antigens. When this complex is purified from tumors, both heat-shock proteins and tumor antigens are obtained. Such an approach has bypassed the requirement of knowledge of the exact tumor antigens when such knowledge is still scarce. Gp96 heat-shock protein – peptide complex (HSPPC-96) – is the best studied of these approaches. Immunization with gp96 peptide complexes led to their uptake by skin DCs through CD91 (a heat-shock protein receptor) followed by cross-presentation of the gp96-chaperoned peptides by the DCs and stimulation of T cells. Phase I/II trials with this approach in human melanoma, renal carcinoma, and colon carcinoma have demonstrated potential clinical activity, though the phase III trial in stage IV melanoma did not show significant survival benefit from this vaccine approach over physicians' choice of treatments. A phase I pilot trial of immunotherapy with autologous tumor-derived HSPPC-96 as an adjuvant therapy for resected pancreatic adenocarcinoma has also been completed. Six weeks after surgery, patients were given HSPPC-96 subcutaneously once a week for 4 weeks. At the time of the report, three of ten treated patients were alive without disease at 2.6, 2.7, and 5.0 years follow-up. Median overall survival was 2.2 years. This study demonstrates the feasibility of preparing HSPPC-96 from pancreatic adenocarcinomas. Nonetheless, autologous anti-HSPPC-96 ELISPOT reactivity increased significantly in only one of five patients examined. There was no observed correlation between immune responses and prognosis [76].

Thus, although personalized multi-antigen vaccine approaches are potentially attractive, they require either resected tumors to purify antigen-containing complexes or peripheral blood to identify reactive peptides. Similar to autologous whole-cell vaccines, such approaches would be less convenient and less reproducible for the clinical practice.

Allogeneic Whole-Cell Pancreatic Vaccines

Both autologous and allogenic vaccine approaches have demonstrated bioactivity in preclinical and clinical studies. Although autologous vaccines would insure that the most immune-relevant antigens are being employed for immunization for a given patient, it is not feasible to obtain enough autologous tumor cells for effective immunization of most patients with pancreatic cancers. Therefore, whole-cell allogeneic vaccines have become an appealing approach since few tumor antigens have so far been identified for pancreatic cancer. Three clinical trials testing an allogeneic GM-CSF secreting tumor vaccine approach alone and in combination with other targeted interventions in patients with resected and metastatic pancreatic cancer have been conducted and completed to date. More than 200 patients have been treated with multiple immunizations, and this approach has been shown to be safe and feasible for patients with all stages of pancreatic cancer. These studies have also demonstrated the safety of the vaccine when given in combination with a number of chemotherapeutic agents and radiation therapy. These studies are summarized below.

The phase I study of an allogeneic GM-CSF-secreting tumor vaccine in patients with resected pancreatic cancer was the first clinical trial to test the hypothesis that allogeneic GM-CSF secreting pancreatic tumor cell lines can prime a systemic immune response in patients with resected pancreatic adenocarcinoma. Fourteen patients with stage 2 or 3 disease received an initial vaccination 8 weeks following resection. This was a dose escalation study in which patients each received 10^7, $5X10^7$, 10^8, and $5X10^8$ vaccine cells. Study patients were jointly enrolled in an adjuvant chemoradiation protocol for 6 months and then given three additional vaccinations 1 month apart at the same original dose that they received for the first vaccination. Toxicities were limited to grade I/II local reactions at the vaccine site and self-limited systemic rashes. Systemic GM-CSF levels were evaluated as an indirect measure of the longevity of vaccine cells at the immunizing site. GM-CSF levels peaked at 48 h following vaccination. The vaccine sites were also evaluated as a measure of the local immune reaction to the vaccine. Eleven of 14 patients demonstrated a local inflammatory response. Postvaccination DTH responses to autologous tumor cells were observed in one of three patients receiving 10^8 and in two of four patients receiving $5X10^8$ vaccine cells [77]. The three DTH responders are the only long-term survivors, and all are still disease-free for more than 10 years. A follow-up phase II study of the GM-CSF-secreting pancreatic tumor vaccine has also been completed at Johns Hopkins Hospital in 60 patients with operable pancreatic cancer. Although the final analysis has not yet been reported, early analysis suggests that there is an overall survival benefit compared with historical controls [78].

The phase I allogeneic vaccine study provided an opportunity to identify candidate targets of the immune response. Immunized lymphocytes from the three disease-free survivors were used in a functional genomic approach to screen genes found to be overexpressed in pancreatic cancers. One gene product, mesothelin, has been reported to serve as a candidate target of T cells responses using this antigen discovery approach. Only the patients demonstrating disease-free survival benefit

also demonstrated a postvaccination induction of mesothelin-specific CD8+ T cells that remained detectable for up to 4 years following treatment without additional boosts [79]. Preliminary data suggests that CD8+ mesothelin-specific T cells are also detected in patients with prolonged disease-free survival in the follow-up phase II study [78].

The GM-CSF secreting, allogeneic vaccine alone and in sequence with immune-modulating doses of cyclophosphamide (Cy) were tested in a phase II study for patients with stage 4 pancreatic cancer who failed gemcitabine-containing chemotherapy. This trial was sponsored by Cell Genesys, Inc., and conducted at both Johns Hopkins Hospital and the US Oncology Group. This was a two-cohort, non-randomized study. Thirty patients in cohort A were administered with vaccines alone; and 20 patients in cohort B- 20 were administered Cy 250 mg/m^2 IV 1 day prior to each vaccination. The results demonstrated that the administration of a GM-CSF-secreting, allogeneic pancreatic cancer vaccine either alone or in sequence with Cy is feasible, safe, and tolerated by patients with advanced pancreatic cancer, the majority of which had received $\geq$2 prior chemotherapy regimens. The median number of vaccines administered was two to patients in cohort A and three to patients in cohort B. Treatment-related adverse events reported in more than 5% of patients included local vaccine injection site reactions, fever, rigors, and rash. Grade 3/4 treatment-related events identified in one patient included leukocytosis, dehydration, and fatigue. Thus, the toxicities (local and systemic) related to the vaccine alone or in sequence with Cy have been low grade and self-limiting. This study represents the first demonstration that integrating immunomodulatory doses of Cy with a GM-CSF-secreting vaccine in patients with advanced pancreatic cancer is safe and feasible to administer. Although this was not a randomized controlled study, stable disease lasting a median of 18 weeks was observed in 16.7% of patients treated by vaccines alone and 40% of patients treated by the combination of vaccines and Cy. Median survival was 2.3 months and 4.7 months, respectively, in a patient population that had received more than two prior chemotherapies. Unlike patients with resected cancers, mesothelin-specific T cell responses were detected at baseline in most patients treated on this study. In addition, there was a trend toward prolonged progression-free survival in those patients who demonstrated persistent and higher avidity mesothelin-specific T cell responses with therapy [80].

Based on early clinical studies, it is possible that allogeneic whole-cell vaccine approaches for pancreatic cancer treatment can bypass the prerequisite for knowing which antigens are dominant pancreatic cancer-associated antigens. However, it should be recognized that identification of dominant pancreatic cancer antigens is still crucial for the assessment of tumor-specific T cell responses and for the future development of multi-antigen targeted vaccine approaches for treatment and possibly prevention of pancreatic cancer. As mentioned above, the whole-cell vaccine approach provides a resource of patient materials for identifying dominant pancreatic cancer antigens. After dominant antigens have been identified, antigen-specific vaccines can be developed to test whether antigen-targeted approaches can induce more specific and more potent antitumor immune responses when the most dominant antigens are delivered in a non-antigen limited formulation. As mentioned above,

vaccines that target multiple specific antigens are the most ideal approach, which further underscores the importance of identifying dominant pancreatic cancer antigens through the use of whole-cell vaccine studies. Antigen-specific vaccines will be discussed in more detail below.

New Strategies for Pancreatic Tumor Whole-Cell Vaccine

Several studies are ongoing to improve on the current GM-CSF-secreting allogeneic vaccine approach. For example, it is still not clear how long to vaccinate following the initial immunizations that were given to patients on the original phase I and II studies conducted at Johns Hopkins. Therefore, a phase II study to evaluate the long-term boosting effect of the GM-CSF-secreting allogeneic pancreatic tumor vaccine is underway. Patients who are eligible for this study are those who have received the same vaccine through prior phase I and phase II trials and remain disease-free since the surgical resection of their primary pancreatic adenocarcinoma. Those who are vaccine naïve may also be eligible for boosters after first receiving four primary vaccinations on a monthly basis. All eligible patients will receive a vaccine boost every 6 months until they have disease progression, or withdraw from the study, or the vaccine source is consumed. The study has recently completed the accrual phase (Laheru et al., personal communication). Mesothelin-specific CD8+ T cell responses are being used to evaluate the induction and maintenance of vaccine-induced immune responses.

As another example, this same allogeneic vaccine approach is being combined with immune-modulating agents to determine if it is possible to improve on the overall outcomes if immune tolerance mechanisms are abrogated. As one example, a phase II trial of the GM-CSF-secreting allogeneic pancreatic tumor vaccine in combination with Erbitux (cetuximab) for the treatment of advanced pancreatic adenocarcinoma is underway at the Johns Hopkins Hospital to test whether cetuximab can enhance the immune priming capabilities of the vaccine. In this study, an immune-modulating dose of cyclophosphamide is also being given 1 day prior to each vaccination to inhibit T regulatory cells (Laheru et al., personal communication). As another example, this same vaccine was given in combination with the anti-CTLA-4 mAb in a pilot study testing the safety and induction of mesothelin-specific CD8+ T cell responses. The study population consists of 30 patients with locally advanced, unresectable, or metastatic pancreatic adenocarcinoma. This is a two-arm study in which 15 patients each received either (1) ipilimumab alone or (2) an allogeneic granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting pancreatic tumor whole-cell vaccine in combination with ipilimumab. Patients receive each treatment every 3 weeks for a total of four induction doses (weeks 1, 4, 7, and 10). At the week 22 evaluation, patients who have had evidence of a response or stable disease were offered the maintenance phase where they received the originally assigned treatment every 12 weeks. Subjects who had early progression followed by stable disease (SD) or better between weeks 14 and 22 were also eligible for maintenance phase treatments. CA19-9

declines in association with the combination treatment of GVAX and ipilimumab were seen for 7/15 patients. In contrast, 0/15 patients receiving ipilimumab alone had CA19-9 declines. Median overall survival (OS) was 3.7 months for arm 1 and 5.7 months for arm 2 ($p = 0.072$). The percentage of patients alive after 1 year also favored the combination arm (7% vs. 27%) [81]. The best RECIST response was SD in two patients in arm 1 and two patients in arm 2. Using the immune-related RECIST criteria, arm 2 had an additional patient with SD for 81 weeks. The quality of the responses in the two arms was different. Patients with SD on arm 1 had continuous disease progression that did not reach the 20% growth cutoff for 7 and 22 weeks. Arm 2 had three SD responses (one patient demonstrated a regression starting at week 14 that was maintained until week 31, another patient's disease stabilized starting at week 22 and was maintained for 81 weeks, and the third SD was maintained for 71 weeks while that patient was on study). This pilot study has supported the combination immunotherapy strategy for pancreatic cancer treatment.

In addition to the allogeneic whole-cell vaccine studies at the Johns Hopkins Hospital, studies with alpha-1,3-galactosyltransferase-expressing allogeneic pancreatic tumor cells represent another allogeneic vaccine. NewLink Genetics Corporation is conducting one phase II adjuvant study of low dose HyperAcute(R)-Pancreatic Cancer Vaccine in combination with chemoradiation and another phase II adjuvant study of standard dose HyperAcute(R)-Pancreatic Cancer Vaccine, both in subjects with surgically resected pancreatic cancer. HyperAcute(R)-Pancreatic Cancer Vaccine is a cancer vaccine comprised of irradiated allogeneic pancreatic cancer cells transfected to express murine alpha-1,3-galactosyltransferase, which results in the expression of murine alpha-1,3-galactosyl (alpha-gal) carbohydrate residues on cell membrane glycoproteins and glycolipids of the vaccine pancreatic cancer cell allograft. Murine alpha-gal epitopes, not present on human cells, induce a hyperacute rejection of the vaccine pancreatic cancer cell allograft. The hyperacute rejection involves the binding of pre-existing human anti-alpha-gal antibodies (which naturally occur against gut flora) to murine alpha-gal epitopes, resulting in the rapid activation of ADCC toward allograft cells. It is hoped that the host immune system will then attack endogenous pancreatic cancer cells, resulting in ADCC toward endogenous pancreatic cancer cells. The phase I trial and phase II trial had demonstrated the safety, feasibility, and potential efficacy of this vaccine in patients with surgically resected pancreatic adenocarcinoma [82]. Nevertheless, the efficacy of this vaccine failed to be substantiated by the phase III study comparing gemcitabine alone and gemcitabine in a sequential combination with the HyperAcute-Panceatic Cancer Vaccine.

Combination Vaccine Therapy

At Johns Hopkins Hospital, a phase I study of recombinant Listeria vaccine targeting mesothelin, which is overexpressed in pancreatic adenocarcinoma as mentioned above, was conducted for the treatment of mesothelin positive advanced malignancies including pancreatic adenocarcinomas. This therapeutic vaccine, CRS207, was

found to be safe and capable of inducing both innate and adaptive immunity to antigens. CRS207 was subsequently tested in sequential combination with GVAX and administrated to patients with metastatic pancreatic cancer. In this phase IIa trial for metastatic pancreatic cancer that progressed through multiple lines of chemotherapy, priming with GVAX followed by boosting with CRS207 was compared to GVAX alone [83]. Overall survival for all patients receiving GVAX + CRS207 was 6.1 months and significantly longer than 3.9 months for those receiving only GVAX. However, the phase IIb study comparing this combination vaccine platform versus CRS207 alone versus single-agent chemotherapy for metastatic pancreatic cancer as a second line or second line above therapy failed to demonstrate that GVAX + CRS207 or CRS207 alone would be superior over single-agent chemotherapy [84]. It should be noted that as many as 40% of the patients who were assigned to the arm of single-agent chemotherapy dropped off the study and the majority of them went to receive combinational chemotherapy regimens. Nevertheless, the GVAX + CRS207 combination vaccine strategy remains valuable as a vaccine platform for further clinical testing, particularly in combination with immune checkpoint inhibitors as described below.

Perspectives

Identification of New Pancreatic Cancer Antigen

The optimal cancer vaccine will target a panel of immune-relevant antigens specific for a given cancer type and be administered in sequence with relevant immune-modulating agents that bypass multiple mechanisms of immune tolerance. Thus, the search for defined pancreatic cancer antigens is still a high priority in the field of tumor immunology. An ideal pancreatic cancer antigen for the vaccine delivery must have the following characters. First, an ideal antigen is one that is specifically expressed by tumor cells relative to normal tissue. A vaccine based on such an antigen may spare the attack of normal cells by vaccine-induced immunity and may be less susceptible to immune tolerance. If mutated gene products are expressed in neoplasm and subsequently neoepitopes are recognized by T cells, they may become neoantigens. A vaccine based on neoantigens is likely less susceptible to immune tolerance. However, the patient's immune system must be tolerant to these neoepitopes; otherwise, it would not have allowed the neoplasm to grow. The major tolerance may lie in the barriers for T cells recognizing neoepitopes to traffic into the tumors. Therefore, an effective immunotherapy must overcome these barriers. Second, as discussed above, it might be a wrong assumption that mutated antigens are not recognized as self-antigen by immune surveillance mechanisms. In fact, non-mutated antigens may have some advantage over mutated antigens. Specifically, a vaccine designed based on a non-mutated antigen may be applicable to many patients, whereas vaccines designed based on a mutated antigen must be individualized. Third, the best antigens, whether mutated or not, should be critical for the development and maintenance of the malignant phenotype. However, an antigen that

is only critical for a certain step in the tumorigenesis process may not be the best target. As discussed above, tumors may develop antigen loss variants once this step of tumorigenesis has occurred.

Identifying candidate pancreatic tumor antigens has been a great challenge. Although there have been a number of genetic- and protein-based approaches that have attempted to identify all types of tumor antigens, few have succeeded. Several approaches have identified a large panel of melanoma-associated tumor antigens. One approach utilized cDNA libraries to T cell clones isolated from growing melanomas. A second approach utilized similar T cell clones to screen antigenic peptides eluted off of HLA molecules and purified by HPLC and mass spectrometry. Although they are important approaches, both had limitations. Specifically, T cell clones were required to identify specific antigens, yet few T cell clones exist against most cancers. Second, these approaches were labor intense and did not necessarily yield generalizable antigens. Finally, both approaches used lymphocytes from patients with actively developing tumors and thus may not have identified the most immune-relevant antigens that serve as the best targets of immune response.

A number of recent approaches that utilize immunized reagents from responding patients and are more rapid methods of antigen identification have been employed to identify pancreatic tumor antigens. One approach uses differential gene analyses to identify genes that are highly expressed in pancreatic cancer relative to normal tissue. As one example, a serial analysis of gene expression (SAGE) approach was employed to identify genes overexpressed in pancreatic cancer [85]. Using this approach and immunized lymphocytes from an allogeneic, GM-CSF-secreting vaccine clinical trial, this group identified mesothelin as a candidate target [79]. Mesothelin is rarely expressed in normal cells, but is highly expressed in several malignancies including near 100% of pancreatic adenocarcinomas. It is a transmembrane glycoprotein member of the mesothelin/megakaryocyte potentiating factor (MPF) family. Recent studies suggested that the function of mesothelin may be important for cancer cell proliferation and migration.

Once a target is identified, it is important to design a series of studies to validate the role of the target as a predictor of who will and who will not respond to a therapy. It is also important to confirm that the target is indeed recognized by immunized T cells. The CD8 T cells from the patients who have received the GM-CSF-secreting allogeneic whole-cell vaccines thus provided materials to evaluate the immunogenicity of mesothelin in pancreatic cancer patients. The immunologic analysis of the patients in the phase I allogeneic pancreative vaccine trial demonstrated that only the three patients who are long-term survivors and also demonstrated a postvaccination DTH response to autologous tumor cells at 28 days following vaccination also demonstrated a postvaccination induction of $CD8^+$ T cells to the mesothelin epitopes predicted by computer algorithms. This difference in detection of mesothelin-specific T cell responses was statistically significant at a $p < 0.001$ by the fisher exact test. These data suggested that it is possible to use mesothelin responses as a predictor of vaccine response in pancreatic cancer patients. In addition, this analysis

demonstrated that $CD8^+$ T cells from one of the patients recognized the natural epitope expressed by the pancreatic tumor [79]. These findings require further validation in larger clinical trials to confirm the value of this antigen as an immune-relevant target of the immune responses.

As discussed above, the identification of mesothelin as a pancreatic tumor antigen has led to the development of mesothelin-specific vaccines, which have been tested in preclinical and clinical studies. As aforementioned, mesothelin is also a target of passive immunotherapy with anti-mesothelin antibodies. These studies and future antigen-targeted studies will provide the critical patient reagents to confirm that mesothelin is indeed an immune target expressed by pancreatic cancers.

A second approach is to take advantage of humoral response in cancer patients and to use their sera to screen antigens. A number of proteomic-based approaches have been used for this purpose. Serological analysis of recombinant cDNA expression libraries (SEREX), serological proteome analysis (SERPA), and protein microarray technology are all being employed. Some tumor-associated antigens were identified by SEREX in the 1990s. With the development and wide application of mass spectrometry technology, SERPA have been used more frequently. Such an approach can employ the vaccine cells themselves as the proteome or other cancer cells. The whole-cell extract from cell lines can be fractioned by techniques such as isoelectric focusing (IFE) and separated by 2D protein electrophoresis (2DE). Immunoblots can then be used to screen immunized sera. Although few pancreatic tumor-specific antigens have been identified using these approaches to date, it is expected that these approaches will identify new immune targets and potential biomarkers in the near future.

Current Developments of Immunotherapy

Focus on Targeting Immune Checkpoints

The current development of immunotherapy is focus on developing strategies that overcome immune tolerance, in particular, strategies that target immune checkpoints (Table 1, Fig. 3). As discussed above, agents that target various mechanisms of immunosuppression have been developed and are currently being investigated in preclinical models or in human studies.

As described above, ipilimumab, a monoclonal antibody that blocks CTLA-4, was approved in 2013 by the FDA for treating advanced melanoma and has become the first immune checkpoint inhibitor indicated for the treatment of cancer diseases. Since 2014, PD-1 and PD-L1 blocking antibodies have been approved by the FDA to treat melanoma, non-small cell lung cancer, renal cell carcinoma, squamous cell carcinoma of head and neck, bladder cancer, and Hodgkin's lymphoma. Immune checkpoint inhibitors as a single agent were shown to induce objective responses in approximately 20–30% of patients with these FDA-approved indications. Half of these responses are durable [86]. However, blocking CTLA-4 and PD-1/PD-L1 as

single therapy has not been effective for pancreatic cancer. How to sensitize pancreatic cancer for immune checkpoint blockade treatments is a priority focus [86].

Focus on Combinatory Therapies

Immune checkpoints targeting agents alone are not ideal treatment strategies for pancreatic cancer and for many other cancers. Pancreatic cancer and checkpoint inhibitor-insensitive cancers are characterized by their immune quiescent tumor microenvironments which are lack of effector immune cell infiltration. On the other hand, vaccines activate tumor-specific T cell immunity and induce the infiltration of effective immune cells. If the vaccine approach is combined with immune modulators, the combinatorial therapy may have a synergistic effect on antitumor T cell activation. Indeed, such a synergy is supported by several preclinical studies. On another hand, the safety of cancer vaccines is supported by most studies that have been so far conducted. The combinatorial therapy is not expected to add any toxicity to that already observed with either agent.

Recently, a study of GVAX given as both neoadjuvant and adjuvant therapy for resectable pancreatic cancer, either alone or with immune-modulating doses of cyclophosphamide to deplete regulatory T cells, was completed [87]. Pathological examination of pancreatic tumor tissue resected just 2 weeks following a single neoadjuvant dose of GVAX identified the formation of novel vaccine-induced, immunologically active, tertiary lymphoid aggregates, organized lymph node-like structures that are not observed in tumor tissue resected from unvaccinated patients. However, activated T cells secrete interferon-γ, which in turn upregulates the PD-1/PD-L1 pathway. These data support an emerging concept that vaccines are required to induce a T cell response that is capable of infiltrating the tumor's microenvironment. However, vaccination is just the first step toward establishing an effective antitumor immune response, converting the pancreatic cancer's tumor microenvironment into an environment similar to what is observed in melanomas exhibiting infiltrating but immunosuppressed T cells prior to immunotherapy treatment. Thus, treatment with GVAX primes the pancreatic cancer's tumor microenvironment for anti-PD-1/PD-L1 antibody therapy. This concept was further supported by demonstrating in a preclinical model of pancreatic cancer that combining anti-PD-1 or anti-PD-L1 antibodies with GVAX enhances the infiltration of effector T cells into pancreatic tumors as well as the cure rate in pancreatic tumor-bearing mice [54, 88]. Thus, vaccination can prime previously unresponsive tumors to become an immune responsive tumor and open the door for novel combination immunotherapies for the majority of immune quiescent tumors.

The above concept has been further developed into a combination immunotherapy strategy, which is being tested in multiple clinical trials for pancreatic cancer including one to test the combination of GVAX + CRS207 and nivolumab for metastatic pancreatic cancer; one to test the combination of GVAX, pembrolizumab, and stereotactic body radiation for locally advanced pancreatic cancer; and one to test the combination of GVAX and nivolumab as neoadjuvant and adjuvant therapy for resectable pancreatic cancer [86].

Optimal Predictors of Antitumor Immune Response

As an immunotherapy does not attack tumor cells directly, but through activating the immune system, it is critical to assess the immune response in every study of immunotherapy. In particular, it is important to ask if the vaccine causes an immune response and if there is any evidence of a clinical response associated with the induced immune response. It might be straightforward to assess the immune response induced by the peptide vaccines, which already have pre-defined immunogenic epitopes. In contrast, it would be more difficult to assess the immune response induced by a whole protein antigen or a whole-cell vaccine. Thus, the recent identification of new targets of the immune response as well as the recent development of new technologies that will allow the identification of new targets in the future should facilitate the assessment of immune-based therapies in treated patients.

As one learns more about immune responses in patients, it is becoming clear that assessing the number of induced T cells is not enough to predict whether a treatment is effective. Traditionally, methods such as ELISA and ELISPOT assays have been used to assess the number and cytokine production of treatment induced T cells. However, more recently, preclinical models have suggested that other parameters such as T cell avidity or potency might be a better predictor of an optimally functioning T cell [89]. The identification of mesothelin as an immune-dominant antigen provides a surrogate marker for immune analysis of the whole-cell pancreatic tumor vaccine. So far, the studies of mesothelin epitopes have also provided an opportunity for better understanding the T cell response in a comprehensive manner. Using dilutional tetramer technology that has recently become available, an analysis of T cell avidity was performed in selected patients from the above described phase II pancreatic GVAX study in metastatic pancreatic cancer. In this pilot study, it was found that the avidity of posttreatment T cells specific for mesothelin epitopes correlated with overall survival. Thus, it will be interesting to assess if the avidity of T cells can serve as an even better predictor of clinical responses in patients receiving adjuvant pancreatic vaccines.

Another challenge that needs to be addressed is whether the analysis of T immune responses targeted at a single antigen can predict who will and will not respond to therapy. Different patient may have different antigen-specific T cell responses depending on a number of factors including the dominant antigens expressed by their tumors and the mechanisms of tolerance that are suppressing the different T cell populations. The same can be said for humoral responses to tumors. It is also not clear which type of responses are most predictive of clinical responses. T cell responses are difficult to analyze with current technologies, especially since it is not clear what parameters of a T cell best predict their activity. Humoral responses, however, are much easier to assess and more standardized. It is likely that analysis of both T cell and humoral responses will require assessing responses to a panel of antigens specific to a given tumor type.

Finally, there is yet an even greater challenge to overcome. The vast majority of studies have examined only T cell or antibody responses in peripheral blood, which

may not always correlate with a patient's response to their tumor. It is very difficult to access most primary or metastatic tumors, especially pancreatic tumors. However, future studies will need to take on this challenge and build in mechanisms by which to sample pancreatic tumors. This is best done by acquiring both pretreatment and posttreatment samples through tumor biopsies. Designing neoadjuvant studies can also allow access to treated tumors. It is important to point out that these patients are providing the scientific community and other patients with the most valuable of resources to better understand this cancer.

Evaluating Clinical Response in the Immunotherapy Studies

It is proposed that cancer vaccines are a therapeutic modality where one should evaluate "patient response" more so than tumor response. It has been argued that standard criteria for tumor response assessment such as RECIST criteria may not be appropriate for the evaluation of immunotherapy. This debate is exemplified by the above described Sipuleucel-T studies did not meet the primary endpoint of disease-free survival, although overall survival was found to be improved by the immunotherapy with a statistical significance [90]. Such a phenomenon has become more common for immunotherapy than anticipated. Immunotherapy does not target tumor cells directly, but through activating the immune system. It is possible that an immunotherapy may induce a durable immune response which eventually suppresses the tumor growth, but is not strong enough in the beginning to cause tumor regression in most of patients. These patients would have been considered to have disease progression and taken off the study based on the RECIST criteria. In addition, it is also possible that some immune-based therapies cause an increase in tumor burden radiographically initially, due to an immune infiltrate, even though the patient feels symptomatically improved. Now, it is known that this is a pseudo-progression that is not uncommon among patients who are receiving immune checkpoint inhibitors [40]. Therefore, immune-related RECIST criteria have been developed for evaluating patients' response in clinical trials of immunotherapy. In some clinical trials, asymptomatic radiographic disease progression may be allowed to keep the patients on study. As long-term clinical outcome are often not predictable early during the course of immunotherapy, how to evaluate clinical responses in immunotherapy studies is still quite challenging and remains to be improved.

Individualized Immunotherapy

Immunotherapy is very different from traditional chemotherapy based on the range of toxicities and the diversity of host responses to the therapy. It is not difficult to conceive that, even to the same tumor, each individual may have a different immune response. It should also be recognized that any of immunotherapy approach may

only be effective in a portion of patients. Thus, the importance of predicting immune responses in the patients receiving immunotherapy is underscored. If a patient's immune response to a particular therapy can be predicted, the next step is to individualize the immunotherapy and design a personalized therapy for this patient. In the future, all immunotherapy studies should be designed toward a defined patient population. One can thus envision that immunotherapy will be customized for delivery to patients based on a panel of antigenic targets specific for a given tumor and on a panel of immune checkpoints that are specific to both the cancer type, cancer stage, and even the individual patient.

Conclusion

Key Research Points

- Recent advances in the tumor immunology field of research have enriched our knowledge of how tumor cells evade immune surveillance and antitumor immunotherapy. Such mechanisms of immune tolerance have been recognized, including:
  - Alterations in T cell signal transduction and cytokine regulation.
  - Tolerance induced by regulatory dendritic cells (DC) and regulatory signals of DC differentiation.
  - Downregulation of co-stimulatory signals.
  - Induction of co-inhibitory signals.
  - Immunosuppressive cells such as regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSC), and tumor-associated microphage.
  - (TAM) altered metabolism in immune cells.
  - The role of the tumor microenvironment in the establishment of immune tolerance is highlighted.

Future Scientific Directions

The future research of pancreatic cancer tumor immunology and immunotherapy should be focused on:

- Identification of new pancreatic cancer antigens
- Development of strategies that target immune checkpoints
- Development of combinatorial therapies of vaccines and immune modulators
- Development of biomarkers for the prediction of antitumor immune responses to new therapies
- Establishment of new criteria to evaluate clinical responses in immunotherapy studies
- Individualized immunotherapy

Clinical Implications

The following strategies of pancreatic cancer immunotherapy are being developed and are under preclinical and clinical testing:

- Monoclonal antibodies
- Adoptive T cell transfer
- Antigen-specific vaccines including peptide vaccines, recombinant vaccines, and dendritic cell-based vaccines
- Allogeneic, GM-CSF-secreting, whole-cell vaccines
- Targeted agents specific for immunologic checkpoints
- Chemotherapy as immune modulators
- The combination of different immunotherapy strategies

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Evolution of Pancreatic Cancer Surgery

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Abstract

Pancreatic cancer surgery has undergone considerable changes during the last decades. While it has been associated with high morbidity and mortality in the 1970s and 1980s, improvements in preoperative diagnosis, perioperative management and surgical techniques have made pancreatic resections highly standardized, safe procedures. Centralization of pancreatic surgery at high volume hospitals has contributed significantly to these developments. In particular, the development of interventional radiology has allowed for non-operative management of complications after pancreatic surgery. Further improvements may be achieved through minimally-invasive approaches using laparoscopy and/or robotic resections. Milestones in long term outcome improvement were the studies of the ESPAC group which demonstrated that adjuvant chemotherapy is highly beneficial for all pancreatic cancer patients, regardless of their tumor stage. The latest ESPAC study demonstrated further improved outcomes with simple adjuvant treatment through a combination of gemcitabine and capecitabine. There has also been exciting progress in the therapy of borderline resectable pancreatic cancer. Neoadjuvant protocols using Folfirinox chemotherapy with or without sequential chemoradiation showed promising response and resection rates. Future studies will assess novel approaches in prevention of postoperative pancreatic fistula rates, in more intensive multimodality treatment and in early diagnosis to improve prognosis.

Keywords

Centralization · Multimodal therapy · Laparoscopic surgery · Robotic surgery · Perioperative management · Precursor lesions

Abbreviations

DGE	Delayed gastric emptying
DP	Distal pancreatectomy
ESPAC	European Study Group for Pancreatic Cancer
HPB	Hepato-pancreato-biliary
PD	Pancreato-duodenenctomy
PDAC	Pancreatic ductal adenocarcinoma
PF	Pancreatic fistula
RCT	Randomized controlled trial

Introduction

Pancreatic cancer surgery has considerably evolved during the last 30 years. Bramhall and colleagues have analyzed treatment and survival in 13.560 patients with pancreatic cancer from 1957 to 1986 from the West Midlands Region Cancer Registry [1]. Two 20 year time periods (from 1957 to 1976 and 1977 to 1986) were analyzed. Resection rates were only 2.6%. Interestingly, 30 day postoperative mortality decreased from 45.2% to 27.6%, whereas 1-year survival increased from 23.8% to 30.6%. Chemotherapy or radiochemotherapy were very rarely used in both periods with 1.9% and 0.9%, respectively. 1-year survival in the overall cohort was 1.3% and

2.0%. These data exemplify the vast progress that pancreatic cancer surgery has made in the past. They show an extremely low rate of resection and of overall survival and that surgery was the only treatment option available. Chemotherapy or chemoradiation were almost never used. Bramhall et al. thus concluded that "the present approach to treating pancreatic cancer in the United Kingdom leaves considerable room for improvement". Interestingly, they also argued that cases suitable for resection should be centralized, e.g. that considerably more cross-referrals should occur than at that time. A similar paper had been published by Michelassi in the Annals of Surgery in 1989 where they described their experience with 647 consecutive pancreatic cancers [2]. They described 90 partial resections and 29 total pancreatectomies. Perioperative mortality was 19% and 5-year survival was low with a total of 14% in all groups. These data demonstrated that surgery was the only hope of cure for pancreatic cancer. However, at that time it was mainly restricted to tumours of the head of pancreas because those of the body and the tail were usually diagnosed late, when metastases had already occurred. Thus, many papers from the 1980s concluded that early diagnosis of pancreatic cancer would be the way to go. Safi and co-authors in 1986 published a paper where they analyzed the importance of CA 19-9 for the prognosis and potential early diagnosis of pancreatic cancer. They demonstrated that 92% of all pancreatic cancer patients had an elevated CA 19-9 of above 37 U/ml, whereas CA19-9 was usually not increased in benign pancreatic diseases. Importantly, they also demonstrated that CA 19-9 was a valid follow-up marker after resection. However they could not clarify whether CA 19-9 was a good marker for early stage carcinoma (stage T1 – T2) [3]. The significant progress made in pancreatic cancer surgery and also in multimodal treatment is best demonstrated when comparing the Kaplan-Meier survival curves from the paper by Michelassi [2]and by the most recent data from the ESPAC trials. While in Michelassi's publication, 5-year survival was 8.8%, current 5-year survival with surgery and adjuvant chemotherapy is 28.8% [4]. The milestones of evolution of pancreatic cancer surgery that have allowed such an increase of 5-year survival rates are the recognition of the importance of centralization of cases, of improved perioperative management and surgical techniques, as well as the more wide-spread use of adjuvant chemotherapy. Most recently, improvement of outcomes for patients with stage 3 and "advanced" stage 2 disease has become a center of interest of international surgical research efforts. Here, novel polychemotherapy regimens in neoadjuvant treatment for locally advanced and borderline resectable cancers offer the hope of further increasing survival rates of these specific subgroups of patients.

Specific Aspects of Evolution in Pancreatic Cancer Surgery

Center Effects

Since the early years 2000s it has become obvious, that centralization of pancreatic cancer surgery significantly improves outcomes after resection. In this respect, John Birkmeyer and collegues have published a landmark paper in the New England Journal of Medicine in 2002 [5]. They analyzed mortality after complex surgical procedures in centers compared to outcomes in regional hospitals. Centers were

defined depending on the number of procedures performed per year. This number depended on the type of procedure performed where for example in colectomy the highest volume centers were defined as those performing more than 124 procedures per year whereas for pancreatic resections high volume centers were defined as those performing more than 61 resections per year. Center effects were most pronounced for complex procedures such as esophagectomies and pancreatic resections. In pancreatic resections, postoperative mortality ranged from 14% to 16% in hospitals where one to two yearly resections were performed to less than 3.8% in those institutions where more than 60 procedures were performed. These data were updated in 2011 and again published in the New England Journal of Medicine [6], demonstrating a decreasing mortality by 19% conferred to a center effect – explained by increased hospital volumes. There were two major reasons underlying these effects, one was that in general more patients were operated on diseases of the pancreas in the US while at the same time there was a centralization of patients to higher volume hospitals. The paper also demonstrated that not only should patients be operated on at high volume hospitals but that at those hospitals outcomes were further improved when high volume surgeons were selected to perform the operation. The most recent publication in this aspect comes from Lidsky and co-authors [7] where 7,806 pancreatic cancer patients were analyzed. Of those, 773 travelled a short distance to a low volume hospital for surgery whereas 758 travelled a long distance to a high volume center to have their pancreatic cancer operation. Thirty and 90 day mortality was significantly lower in the long travel high volume hospitals with two versus 6.3% and 6.5% versus 11.3%, respectively. In addition, the authors demonstrate that the rates of negative margin resections were lower in the high volume hospitals. These effects also translated into long term survival were travelling a long distance to a high volume center was beneficial for overall survival. The authors conclude that these data support ongoing efforts of centralized care for patients undergoing pancreatic cancer surgery.

In conclusion, centralization of pancreatic cancer surgery has had an enormous effect on short and long term outcomes and efforts will be necessary to further improve outcomes by centralizing pancreatic cancer care.

Adjuvant Therapy

One of the most important groups that significantly contributed to the improvements in pancreatic cancer outcomes is the European Study Group for Pancreatic Cancer (ESPAC). Founded in 1992, its initial aims are still valid with the development of an international scientific exchange forum, of combination and consolidation of current pancreas cancer research and the management of collaborative research efforts. As described above, there was no real standard of adjuvant therapy for resectable pancreatic cancer in the early 1990s. The ESPAC group thus developed the ESPAC-1 trial where 541 patients were randomized (in a 2×2 factorial design) into the following adjuvant treatment arms: chemotherapy versus no chemotherapy, chemoradiation versus no chemoradiation [8]. In essence, this landmark study

demonstrated that adjuvant chemotherapy significantly increases survival as compared to no chemotherapy. Interestingly, survival was worse with chemoradiation than with no chemoradiation. This has led to a change of clinical practice (at least in Europe), where adjuvant chemotherapy has become standard while adjuvant chemoradiation has been almost universally abandoned. The next step was the ESPAC-3 trial which compared adjuvant chemotherapy with 5-FU (the "novel" standard from the ESPAC-1 trial) with gemcitabine. Here, 5-FU and gemcitabine were shown to be equally effective [9]. The most recent ESPAC-4 study compared adjuvant gemcitabine with gemcitabine plus capecitabine, demonstrating even better survival in the combination chemotherapy arm. Median survival with gemcitabine and capecitabine was 28 months, compared to 16 months with gemcitabine alone [4]. Thus, the current standard of care in adjuvant pancreas cancer treatment should be this combination chemotherapy protocol. These data again exemplify the enormous progress that has been made in pancreatic cancer treatment and the importance of international collaborations to achieve the goal of performing high-quality multicentre studies with the aim to create evidence and practice-changing results.

Neoadjuvant Approaches

Neoadjuvant treatment for pancreatic cancer has been carried out in an extremely low number of patients for a long time. There were several reasons for this, of which the main reason was that there was no effective chemotherapy or chemoradiation to allow for downstaging of the tumor. With the advent of the polychemotherapy regimen with Folfirinox [10], many efforts started to evaluate the efficacy of Folfirinox in patients with borderline resectable or locally advanced pancreatic cancer. Very recent data from several institutions world-wide [11–14] have demonstrated response rates of up to 60% with FOLFIRINOX and of about 50% with Gem-Abraxane (Gemcitabine plus albumin-coupled Paclitaxel). Pre-treatment with Folfirinox has also revitalised extensive surgical approaches with arterial resections and reconstructions, which had been almost abandoned for a long time because of high mortality and low oncological effectiveness [15]. After pre-treatment however, quite some groups have demonstrated that negative margins can be achieved with extensive resections [16], including the hepatic or superior mesenteric artery – and more often than not, also the superior mesenteric/portal vein. Further research into outcomes after these very specific and aggressive resections is necessary to define their value for patients with stage 3 or advanced stage 2 disease (after neoadjuvant treatment).

Laparoscopic Distal Pancreatectomy

Laparoscopic and robotic approaches are increasingly performed for all indications in pancreatic surgery [17]. DP as the most commonly performed type of minimally invasive resection is regarded as a standard today although concerns regarding oncological radicality are still discussed controversely and there is still no high-level

Table 1 Series on laparoscopic distal pancreatectomy for pancreatic ductal adenocarcinoma with reports on oncological outcomes

	No. of patients				
	Total	PDAC (n, %)	No. of lymph nodes	R0 rate	Tumor size(mm)
Song et al. [23]	359	34(9.5)	10.3	92%	30
Gagner et al. [24]	82	18(22)	14.5	90%	53
Marangos et al. [25]	30	28(93)	5	93%	50
Taylor et al. [26]	46	10(22)	nm	100%	nm
Melotti et al. [27]	58	7 (12)	13	100%	35
Asbun et al. [28]	29	5 (17)	14 (19)	97%	nm
Edwin et al. [29]	17	4 (24)	nm	88 (50)	28
Dulucq et al. [30]	21	3 (14)	18	100%	42
Bärlehner et al. [31]	5	2(40)	19/6	R0/Rx	nm
Sa Cunha et al. [32]	31	1(3)	nm	100%	37
D'Angelica et al. [33]	16	1(6)	5.5	77%	40

nm not mentioned

evidence that laparoscopic DP offers advantages although available studies have shown that it may be superior to open DP in terms of blood loss and hospital stay [18]. As laparoscopic DP was initially preferred in benign indications and small pancreatic lesions offering a technically easy operation [19]. Consequently, the available data on laparoscopic DP for PDAC are still limited but steadily increasing. One of the earliest studies [20] was published by Patterson and colleagues in 2001, demonstrating morbidity rates comparable to open surgery series but a shorter length of hospital stay. The authors concluded that laparoscopic distal pancreatectomy appears to be safe for benign diseases. A large number of mainly single-center experience reports followed, of which Table 1 shows an overview [21, 22]. Because of an increasingly standardized and internationally comparable technical approach, recent research has focused on extending the indications for laparoscopic distal pancreatectomy, on improving oncological outcomes and on analysing cost-effectiveness. The most recent Cochrane meta-analysis confirmed that laparoscopic distal pancreatectomy seems to be beneficial in many aspects but that data quality is low and that randomised trials are urgently needed to better define the value of the minimally-invasive approach [18].

Laparoscopic Pancreaticoduodenectomy

Minimally-invasive PD for PDAC has not gained widespread acceptance yet, however, the numbers of this procedure are increasing. However, it is a technically demanding operation with a considerable learning curve. In addition, experience with advanced laparoscopy is almost mandatory but is not yet reflected in the curricula of most of the HPB fellowship programs. Thus, only few surgeons worldwide have accumulated relevant numbers of laparoscopic pancreato-duodenectomies

Table 2 Studies on open and laparoscopic pancreato-duodenectomy including ≥30 patients

	Type of PD	No. of patients	Operation time	PF	DGE	Mortality	Hospital stay
Wellner et al. [34]	Open	40	410 min	28% (B/C)	28% (B/C)	0	16 days
	Lap.	40	343 min	18% (B/C)	13% (B/C)	2.5%	14 days
Dokmak et al. [35]	Open	46	264 min	41%	15%	0	25 days
	Lap.	46	342 min	48%	17%	2.1%	23 days
Tan et al. [55]	Open	30	372 min	20%	10%	3.3%	12 days
	Lap.	30	513 min	33%	7%	0	10 days
Mesleh et al. [57]	Open	48	355 min	6% (B/C)	8%	nm	8 days
	Lap.	75	555 min	9% (B/C)	13%	nm	7 days
Croome et al. [57]	Open	214	388 min	12% (B/C)	18% (B/C)	1%	9 days
	Lap.	108	379 min	11% (B/C)	11% (B/C)	2%	6 days

nm not mentioned

(Table 2). In such centers, outcomes of open and laparoscopic Whipple procedures are comparable in terms of safety of the procedure and of postoperative morbidity. However, it has to be noticed that in most series, considerably longer operation times are reported and laparoscopic procedures are associated with higher costs in terms of technical devices. How far these disadvantages can be compensated by an enhanced postoperative recovery of the patients and a potentially shorter hospital stay remains unclear as the currently available studies show trends but allow no definite conclusions (Table 2). Furthermore, long term oncological outcomes are not clear and further analyses are required to define whether oncological results will be adequate as well as to confirm the above-mentioned potential advantages (i.e. in terms of blood loss or shorter hospital stay) in randomized controlled trials which are not available to date.

Robotic Resections

While robotic distal pancreatectomy is performed at quite some centers world-wide (Table 3), only a few surgeons have adopted robotic pancreato-duodenectomy. However, there are some institutions where this procedure is performed at significantly increasing numbers and where outcomes are comparable to open surgery. Most recently, a multi-institutional analysis from the US compared results of open and robotic pancreato-duodenectomy [36]. The authors performed a multivariable analysis that demonstrated longer operative times with robotic resections, but reduced blood loss and less major complications. Mortality, rates of clinically relevant pancreatic fistula rates, length of hospital stay and readmission rates were comparable. In terms of oncological radicality, the operative approach was no predictor of margin status or the number of lymph nodes harvested. However, long term data of outcomes after robotic pancreaticoduodenectomy for pancreatic cancer

Table 3 Series of robotic distal pancreatectomy

	Hwang et al. [37]	Daouadi et al. [38]	Waters et al. [39]	Giulianotti et al. [40]	Kang et al. [41]
Duration (year)	2007–2011	2004–2011	2008–2009	2000–2007	2006–2010
Location	Seoul (South Korea)	Pittsburgh (USA)	Indianapolis (USA)	Chicago (USA), Grosseto (Italy)	Seoul (South Korea)
No. of Patients	22	30	17	77in Italy, 57 in the US	20
Tumor size (cm)	3.2 ± 1.5	2.6 ± 1.4	2 ± 1	2.1in Italy and 3.6 in the US	3.5 ± 1.3
LOS	7.0 ± 2.4	6.1 ± 1.7	5.7	21.8 in Italy and 9.3 in the US	7.1 ± 2.2
PF	2(9.1%)	14 (46.7%)	0	36(46.8%) in Italy and 24(42.1%) in the US	nm
Converted to open	0	0	2 (11.7%)	10(13.0%) in Italy, 4(7.0%) in the US	nm

nm not mentioned

is still missing. These data will be of enormous importance to define the value of robotic resections in pancreatic surgical oncology.

Perioperative Management

The evolution of perioperative management is characterized by efforts to improve routine procedures and to create evidence in randomized controlled trials. Important achievements in this field include routine preoperative biliary drainage for jaundiced patients, routine intraoperative drain placement as well as nutritional management and complication management.

Pre-operative Biliary Drainage

For many years, pre-operative biliary drainage in jaundiced pancreatic cancer patients had been an intensively discussed issue. Some studies suggested that all patients should have biliary stenting to relieve jaundice while others suggest to drain almost no patient – unless there was significant impairment of liver function or the presence of clinically relevant cholangitis. In 2010, a randomized trial on biliary drainage versus no drainage was published in the New England Journal of Medicine, demonstrating that routine biliary stenting in patients with bilirubin levels less between 2.3 and 14.6 mg/dl was associated with significantly more postoperative infectious complications (mainly wound infections) than in the group of patients who underwent surgery right away [42]. This paper has changed clinical practice in

that routine pre-operative biliary drainage in jaundiced patients should not be performed anymore. However, it is mandatory to proceed with surgery as quickly as possible. Because this is not possible in many health systems world-wide, biliary stenting still is performed at considerable numbers. A future question will thus be whether prophylactic antibiotic treatment will be of value in this particular group of patients.

Placement of Intra-abdominal Drains in Pancreaticoduodenectomy

In most centers, the routine placement of intra-abdominal drains is performed for every type of pancreatic resection. For PD, this approach has recently been challenged on the basis of two randomized trials [40, 43]. Both studies showed that a routine drainage placement is not beneficial in terms of complication prevention and consequently a selective drainage use should be preferred. In contrast, another trial published in 2014 [44], showed a negative effect of drain omission with an increase in severe postoperative morbidity and even mortality. However, the findings of this trial may be interpreted with caution as the multicenter setting may have caused a bias. Most recently, an RCT from several German centers [43] clearly demonstrated that there is no need for routine placement of drains in pancreaticoduodenectomy. In particular, there were comparable numbers of re-interventions, of in-hospital mortality and morbidity. Rates of clinically relevant postoperative pancreatic fistula and fistula-associated complications were significantly lower in the no-drain group. In conclusion, there is level 1 evidence that no drains should be placed in pancreaticoduodenectomy unless very specific conditions may force the surgeon to do so.

Management of Postoperative Complications

Pancreatic surgery has shown a significant decrease in postoperative morbidity and mortality during the last decades. Historic mortality rates ranging between 20% and 50% have continuously decreased which is not only attributed to surgical progress but also to developments in complication management. Although rates of postoperative pancreatic fistula still remain relatively high, this potentially life-threatening event has been turned into a manageable complication in most patients [45]. A variety of factors has contributed to the enormous reduction in fistula-associated deaths. First and foremost, advances in interventional radiology have allows for a reduction of re-operations which were (and are still) associated with considerable mortality. The most important intervention is the CT-guided drainage of postoperative fluid collections around the pancreatic anastomosis – which are in the vast majority of cases a result of a pancreatic fistula – combined with effective antibiotic therapy [46]. In addition, postoperative haemorrhage as a result of a persisting pancreatic fistula has become a much rarer complication; and, once it occurs, this problem can frequently be dealt with through interventional coiling or stenting [47]. More and more experience with complex cases in centers with high number of yearly

cases has also allowed for much earlier recognition of postoperative complications [48]. Consequently, modern PDAC surgery is shifting towards extended resections, including vascular resections as well as multivisceral approaches, as even these operations can be performed with good perioperative results and are feasible in experienced hands today.

Elderly Patients

While advanced age has been considered a contraindication to extensive pancreatic surgery for a long time, many recent reports demonstrate that pancreatic resections can be performed safely even in octogenarians [49, 50]. However and as with younger patients, co-morbidities need to carefully weighed against the potential benefit conferred by resection of the tumor [51]. If these precautions are not taken pre-operatively, morbidity and mortality tend to be significantly higher in patients older than 80 years.

Resection of Precursor Lesions

One of the most exciting fields in pancreatic surgery in the last decade has been the evolution of treatment of cystic lesions of the pancreas. Because of higher resolution and more widely available imaging, the incidence of pancreatic cysts (mainly IPMNs) has increased dramatically [52]. This is particularly challenging because the vast majority of these cystic lesions are benign, while at the same time, these lesions are the only clinically relevant precursors of pancreatic cancer. Thus, surgical resection of the pre-malignant proportion of these cysts allows for the first time for true early diagnosis and treatment of pancreatic cancer; this is in turn the only truly curative approach for a subgroup of patients (not yet) suffering from this disease – and could thus be called prophylactic pancreatic cancer surgery. However, there are a large number of obstacles. Firstly, a clear pre-treatment diagnosis of the exact cystic entity can often not be achieved. This holds particularly true for side branch IPMN which are the most common (suspected) entity found on cross-sectional imaging. Secondly, it remains very difficult to judge the malignant potential of many of the cystic lesions that are usually incidentally detected; unless clear features for a high risk of malignancy are identified. Such features have been classified in the most recent consensus criteria for IPMN, called the Fukuoka criteria. Here, a group of worrisome features and of high risk stigmata were defined, where the latter carry a high enough risk of malignancy to justify immediate resection [53]. Besides this consensus statement, the European guidelines for the management of cystic pancreatic lesions [54] have approached the management of these precursor lesions. Despite these publications, there is ongoing controversy regarding the timing and extent of surgery, especially in branch duct IPMN and this is one of the most evolutionary fields at the moment and the potential to prevent pancreatic cancer development by precursor resection at the correct point of time is a unique chance to improve the fatal prognosis of this disease.

Conclusion

In the last two decades, pancreatic cancer surgery has become an instrumental and central part of abdominal surgical oncology. While it had once been associated with unacceptably high rates of morbidity and mortality, highly standardized surgical techniques and tremendous advances in perioperative management have allowed to make these resections very safe and effective procedures. Certainly, the advancement of interventional radiology has greatly contributed to this development. Adjuvant treatment has also been transferred from an experimental approach to daily clinical practice and has significantly increased survival in the group of resected pancreatic cancer patients. Neoadjuvant treatment with multimodality regimens or with poly-chemotherapy seems to have similar effects in the group of patients deemed unresectable for a long time. Further detailed research will however be necessary to determine the true value of pre-operative treatment in pancreatic cancer. Resection of cystic lesions and in particular of IPMN has for the first time allowed for resection of a precursor of pancreatic cancer. As with neoadjuvant treatment, many areas of uncertainty remain and international, multi-center efforts will be required to operate on more patients with potentially malignant cystic lesions while not operating on those with truly benign cysts.

Cross-References

- ▶ Adjuvant Chemotherapy in Pancreatic Cancer
- ▶ Clinical Decision-Making in Pancreatic Cancer
- ▶ Interventional Radiology for Pancreatic Cancer
- ▶ Laparoscopic Surgery for Pancreatic Neoplasms
- ▶ Neoadjuvant Chemotherapy in Pancreatic Cancer
- ▶ Surgical Resection for Pancreatic Cancer using the International Study Group of Pancreatic Surgery (ISGPS) Classifications

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Multiparameter Modalities for the Study of Patients in the Setting of Individualized Medicine

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Abstract

The recent revolution in cancer genetics offers the promise of using genetic information to individualize patient treatment. In pancreatic cancer, numerous studies have described a genetic landscape characterized by a set of commonly mutated genes aggregated into core molecular pathways accompanied by numerous but infrequently mutated genes. Studies have also demonstrated significant intratumoral heterogeneity. Resistance against chemotherapeutic agents has also been attributed to difficulty of drug delivery through a rich stromal microenvironment. For these reasons, therapeutic development against pancreatic cancer has been challenging, and a number of promising agents have failed clinical trial testing. Personalized models have been studied as a tool for testing candidate drugs to select the most efficacious treatment. The patient-derived xenograft (PDX) is a well-established preclinical tool to improve the drug screening and development. The PDX model requires adequate tissue for transplantation, and failure is common. A recently described, innovative three-dimensional organoid culture platform can be exploited for genomic and functional studies at the level of the individual patient for personalized treatment approach. Organoid technology may fill the gap between cancer genetics and patient trials and allow personalized therapy design. Combination of genome-based medicine and individualized model-based drug screening may fulfill the promise of precision medicine for pancreatic cancer.

Keywords

Precision medicine · Three-dimensional organoid culture · Patient-derived xenograft (PDX) · Genomic-based medicine

Introduction

The field of oncology is rapidly evolving from treating large, unselected populations to targeting small numbers of patients using deep evaluation of molecular features and selection of the most appropriate treatment. President Obama announced the launching of a Precision Medicine Initiative in his 2015 State of the Union Address, and he requested 215 million dollars to fund this endeavor in the fiscal year 2016. The time is right to pursue this strategy, using the individual patient's genetic information to guide individualized therapy. The significant revolution in cancer genetics is allowing, for the first time, the gathering of enormous amounts of genomic information, including the assessment of complete cancer genomes, to aid in clinical decision-making. From this approach, numerous potential targets have emerged for individual patients that may potentially be linked to clinical response.

Genomic-based treatment has already provided examples of remarkable success stories. The development of Imatinib to treat CML and GIST, BRAF inhibitors to treat melanoma, HER2 antibodies to treat HER2 positive breast cancer, and EGFR inhibitors and ALK inhibitors to treat nonsmall cell lung cancer are just some

examples that have dramatically changed the treatment paradigms and improved the survival of patients.

Targeted therapy development continues to evolve rapidly, and this approach has intuitively expanded to precision medicine. NCI-Molecular Analysis for Therapy Choice (NCI-MATCH) (ClinicalTrials.gov number, NCT02465060) is a clinical trial to treat cancer patients according to their molecular abnormalities using DNA sequencing from biopsy specimens. The drugs included in this trial are approved by US Food and Drug Administration (FDA) for another cancer indication or are being tested in clinical trials and have shown a promising result against solid tumors such as breast, colon, lung, prostate, or lymphoma with specific mutations. The AURORA clinical trial is expected to establish detailed molecular profiling of metastatic breast cancer for deeper understanding of the molecular biology, promising to lead to personalized cancer medicine (ClinicalTrials.gov number, NCT02102165).

Pancreatic ductal adenocarcinoma (PDA) remains one of the most deadly cancers worldwide, with 5-year survival below 7%. Surgical resection, the only potentially curative treatment for PDA, is performed in only 15 to 20% of PDA patients, as most cases are diagnosed at a late stage when surgery is not possible. Recent advances in chemotherapy, such as development of the FOLFIRINOX (5-fluorouracil, leucovorin, irinotecan, and oxaliplatin) regimen, and gemcitabine and nanoalbumin-bound paclitaxel, have extended the survival of PDA patients. Although other types of cancer patients are treated based on specific markers, there are no effective markers for targeted therapy in PDA.

The epidermal growth factor receptor (EGFR) inhibitor, erlotinib, the only FDA approved targeted agent for treating PDA, only marginally extends overall survival in combination with gemcitabine. Poly-ADP-ribose polymerase (PARP) inhibitors have shown promising preliminary results [1]. This agent was first reported for BRCA1/2 positive breast cancer and ovarian cancer.

Recent expression analysis has identified four molecular subtypes of PDA [2]. This and other integrated molecular analyses are expected to give insights with therapeutic relevance. One hypothesizes that treatments could be individualized based on a patient's molecular subtype. For example, immune modulators could be tested in patients with an immunogenic subtype. In terms of precision treatment, categorizing some specific patients according to active, available drugs is a logical way forward. Recent clinical trials have shown the efficacy of PARP inhibitor for patients with BRCA1/2 or PALB2 mutations [3, 4]. The frequency of BRCA1/2 deficiency is 5–8% in the general population and 12–15% in certain groups such as Ashkenazi Jewish patients with a family history of breast cancer. Patients with BRCA deficiency driven tumors have increased sensitivity to platinum agents. In addition to platinum agents, BRCA deficient cancers have shown high sensitivity to PARP inhibitors. Recent sequencing data suggest that mutations in BRCA pathway component genes and surrogate measures of defects in DNA maintenance (genomic instability and the BRCA mutational signature) have potential implications for therapeutic selection for PDA in the absence of BRCA or PALB2 mutations [5].

Personalized medicine for PDA patients will be based on an enhanced understanding of biological features of PDA, advancement of technology, and treatment development. Advances in technology currently allows for faster and less expensive whole-genome, exome, and transcriptome analyses compared with traditional Sanger-based methods, enabling routine and rapid characterization of genetic and pathway alterations. Some trials are already underway to test this concept. In the IMPaCT (The Individualized Molecular Pancreatic Cancer Therapy) trial [6], HER2 amplification, KRAS wild-type, and mutations in DNA damage repair pathways (BRCA1, BRCA2, PALB2, ATM) are assessed for guiding treatment. Another approach utilizes the patient derived xenograft (PDX) mouse model, a so-called avatar model. The PDX represents a valuable preclinical tool for studying human cancer biology and patient response to treatments, which suggest the potential for precision medicine. Due to the short survival seen in PDA, participants of clinical trials are often unable to be treated according to their molecular analysis due to their worsening conditions or progression of their disease. For precision medicine to be effective in PDA, developing rapid analyses is a prerequisite.

Genetic Screening and Genomic-Based Treatment

Based on rigorous molecular pathology studies and genomic analyses, the generally accepted model of carcinogenesis describes a stepwise progression from normal pancreatic epithelia to pancreatic intraepithelial neoplasia (PanIN) and finally to frank adenocarcinoma with accumulation of accompanying signature mutations. Recent genomic analyses of PDA have revealed a complex mutational landscape [2, 5, 7]. More than 90% of PDA carry activating KRAS mutations. Mutations in KRAS are seen in all stages of PanIN. Inactivation of tumor suppressor genes such as TP53, Smad4, and p16 are seen with progressive PanIN development and occur at rates of more than 50%. The prevalence of recurrently mutated genes then drops to ~10% which aggregate into core molecular pathways including KRAS, WNT, NOTCH, DNA damage repair, RNA processing, cell cycle regulation, TGF-β signaling, SWI-SNF, chromatin regulation, and axonal guidance. For a number of reasons, including inter- and intra tumor heterogeneity, and an inability to target commonly mutated genes, development of targeted and effective therapeutics remains challenging.

Jones et al. [8] reported a core set of 12 cellular signaling pathways altered in PDA, including apoptosis (100%), DNA damage control (83%), regulation of G1/S phase transition (100%), hedgehog signaling (100%), homophilic cell adhesion (79%), integrin signaling (67%), c-Jun N-terminal kinase signaling (96%), KRAS signaling (100%), regulation of invasion (92%), small GTPase–dependent signaling (other than KRAS) (79%), TGF-β signaling (100%), and Wnt/Notch signaling (100%). Jones and colleagues determined the sequences of 23,219 transcripts, representing 20,661 protein-coding genes and found that PDA contains an average of 63 genetic alterations, the majority of which are point mutations. They collected

24 PDA DNA samples from 10 PDXs and 14 cell lines from 17 patients with surgically resected and 7 patients who underwent a rapid autopsy. Normal tissues were obtained from tumor-negative duodenum, liver, or spleen. These 12 pathways are genetically altered in the great majority of pancreatic cancers. However, the pathway components that are altered in any individual tumor vary widely and the specific genes altered in each tumor are largely different. In addition, it is difficult to determine whether each identified mutation plays a functional role in the pathway or process identified.

Biankin et al. [7] performed exome sequencing and copy number analysis of early (stage I and II) PDA. Biankin and colleagues identified substantial heterogeneity with 2016 nonsilent mutations and 1628 copy-number variations from the analysis of informative 99 tumor samples. They defined 16 significantly mutated genes, reaffirming known mutations (*KRAS*, *TP53*, *CDKN2A, SMAD4*, *MLL3*, *TGFBR2, ARID1A,* and *SF3B1*), and uncovered novel mutated genes including additional genes involved in chromatin modification (*EPC1* and *ARID2*), DNA damage repair (*ATM*), and other mechanisms (*ZIM2*, *MAP 2 K4*, *NALCN*, *SLC16A4,* and *MAGEA6*). Pathway-based analysis of recurrently mutated genes identified mechanisms known to be important in cancer: G1/S checkpoint machinery, apoptosis, regulation of angiogenesis, and TGF-β signaling. They identified frequent and diverse somatic aberrations in genes described traditionally as embryonic regulators of axon guidance, particularly SLIT/ROBO signaling which suggested the potential involvement of axon guidance genes in pancreatic carcinogenesis.

Bailey et al. [2] reported that mutated genes aggregated into 10 molecular mechanisms, including activating mutations of KRAS in 92%; disruption of G1/S checkpoint machinery (TP53, CDKN2A, and TP53BP2) in 78%; TGF-β signaling (SMAD4, SMAD3, TGFBR1, TFGBR2, ACVR1B, and ACVR2A) in 47%; histone modification (KDM6A, SETD2, and ASCOM complex members MLL2 and MLL3) in 24%; the SWI/SNF complex (ARID1A, PBRM1, and SMARCA4) in 14%; the BRCA pathway (BRCA1, BRCA2, ATM, and PALB2: 5% germline, 12% somatic); WNT signaling defects through RNF43 mutation (5%); and RNA processing genes, SF3B1, U2AF1, and RBM10 (16%).

Genomic instability is a characteristic feature of almost all human cancers. Germline mutations in DNA mismatch repair (MMR) genes have been reported in hereditary cancers. With regard to the molecular basis of genomic instability in sporadic cancers, recent genome-wide studies by the use of Sanger sequencing reported that mutations in DNA repair genes and mitotic checkpoint genes were infrequent. Wang et al. sequenced the exomes of 15 human PDA-derived cell lines and their matched normal samples and identified a total of 1517 somatic mutations. Among them, 56 genes were recurrently mutated in two or more cell lines and showed dramatically increased rate of both indels and substitutions involved in all nine core signaling pathways. They revealed that MLH1 expression levels appear to be correlated with the mutation rates. Among the MMR proteins, the loss of MLH1 is the most common cause of MSI [9].

Epigenome

While a significant effort has been made to understand the somatic genetic alterations acquired in PDA, research into epigenetic mechanisms has expanded our understanding of altered gene expression in PDA. Research has focused on several well-characterized epigenetic mechanisms, including DNA methylation, histone modification, and microRNAs. It is increasingly understood that multiple epigenetic mechanisms are indeed crucial in the development and progression of PDA. In addition to genetic changes, epigenetic alterations add another layer of complexity and contribute to the heterogeneity of PDA.

Studies on chromatin dynamics alone are unveiling the existence of robust machineries that can mediate epigenetic changes in pancreatic cells. These findings highlight the need to further our insight into how epigenetic mechanisms are able to independently and cooperatively influence gene regulation and thereby PDA development.

Furthermore, it is important to emphasize one of the characteristics of epigenetic mechanisms of gene regulation – their reversibility. This feature provides a unique target for the introduction of specific therapeutic interventions for PDA.

Nones et al. reported a large-scale methylation and expression profiling study of 167 PDA compared with 29 adjacent nonmalignant pancreas. A total of 11,634 CpG sites associated with 3522 genes and pathway analysis revealed an enrichment of aberrantly methylated genes involved in core signaling pathways including TGF-β, WNT, integrin signaling, cell adhesion, stellate cell activation, and axon guidance. Notably, they revealed epigenetic suppression of SLIT-ROBO signaling and upregulation of MET and ITGA2 expression, which is correlated with poor outcome. Biankin et al. identified genomic aberration of ROBO1 in 11% and SLIT in 10% of PDA samples. Nones et al. suggested that hypermethylation of SLIT-ROBO is a more widespread mechanism of inactivation of this pathway. From the 58 tumors 48% showed hypermethylation of all four genes (ROBO1, ROBO3, SKIT2, and SLIT3). Tumor suppressor genes with a low incidence of mutations may be inactivated by epigenetic mechanisms more frequently. DNA methylation cooperating with other genetic mechanisms alter key signaling pathways critical to cancer development [10].

Chromatin regulators such as HDACs and BET proteins are currently being analyzed as potential strategies for PDAC patients [11, 12].

Transcriptomic PDA Subtypes

Treatment outcomes are improved by targeting drugs according to tumor subtypes in other cancers. Identification of therapeutic molecular subtypes in PDA has been challenging. Collisson et al., for the first time, demonstrated three gene expression subtypes using a 62-gene signature (PDAssigner; [13]) applied to laser capture–microdissected epithelial PDA tumors. They designated these subtypes as classical, quasimesenchymal (QM), and exocrine-like. Classical PDA [14] is

characterized by high adhesion-associated ribosomal and epithelial gene expression, and elevated GATA6 expression, which is essential for pancreatic development [13]. QM-PDA showed high expression of mesenchymal-associated genes. Exocrine-like PDA shows high expression of tumor cell–derived digestive genes. However, in 19 human and 15 mouse PDA cell lines, only the classical and the QM-PDA subtypes were identified, suggesting that currently used PDA cell lines inadequately represent the heterogeneity of human PDA. They showed that classical PDA lines are relatively more dependent on Kras and more sensitive to erlotinib than QM-PDA lines. Conversely, QM-PDA lines are more sensitive to gemcitabine than classical PDA. However, the drug sensitivity of the exocrine-like subtype has yet to be determined.

The presence of the exocrine-like subtype was validated by Noll and colleagues [15], by deriving matched exocrine-like PDA patient-derived xenograft tumors and cell lines. In addition, they showed that the exocrine-like PDA subtype is resistant to small-molecule drugs dasatinib, erlotinib, and paclitaxel and that this resistance is mediated by a cell-autonomous CYP (cytochrome P450) 3A5-dependent drug detoxification mechanism. CYP3A5 also contributes to acquired drug resistance in other subtypes of PDA and in other malignancies.

They identified the subtype by two surrogate markers, HNF1A for exocrine-like PDA and KRT81 for QM-PDA. Classical PDA was defined as double negative of these markers. HNF1A+ cases are more differentiated whereas KRT81+ cases are less differentiated. Exocrine-like PDAs were found to have the best survival rates.

Moffitt et al. [14] identified two tumor subtypes as classical and basal-like and two stromal subtypes as normal and activated by digitally separating tumor, stromal and normal gene expression. The Collisson classical and QM subtypes appeared to be a mixed collection of genes from the Moffitt basal-like and stromal subtypes. Although the basal-like tumor subtype, which is molecularly similar to basal tumors in bladder and breast cancers, demonstrated worse outcomes, basal-like tumors showed better response to adjuvant therapy. The activated stromal subtype showed worse prognosis than normal stromal subtype. The KRAS mutation encoding G12D was associated with basal-like subtype, and the KRAS-G12 V allele was higher in African Americans. In addition, Collisson and colleagues demonstrated high inter-patient tumor heterogeneity and low heterogeneity between primary and metastatic sites.

Bailey et al. [2] demonstrated four subtypes of PDA using RNA-sequencing data from 96 bulk tumors with high epithelial content. They named these subtypes squamous, pancreatic progenitor, immunogenic, and aberrantly differentiated endocrine exocrine (ADEX). These four subtypes were associated with specific histological characteristics. Squamous showed adenosquamous carcinoma, pancreatic progenitor and immunogenic showed mucinous noncystic (colloid) adenocarcinoma and carcinoma arising from IPMN, and ADEX showed acinar cell carcinoma. Three of four subtypes overlap with the Collisson subtypes with the exception of immunogenic subtype. The Collisson QM, classical, and exocrine-like subtypes correspond to the Bailey squamous, pancreatic progenitor, and ADEX subtypes, respectively. The immunogenic class shares many of the characteristics of the pancreatic progenitor class but is uniquely associated with a significant immune cells infiltration.

Proteomics

Proteomics research offers the promise of discovering biomarkers for improvement of early diagnosis and prediction of response to therapy. Several candidate protein biomarkers have been investigated to date. Unfortunately, many of these biomarkers are not specific for PDA or in situ lesions, as they are detected in patients with pancreatitis and other conditions such as smokers. Examples include carbohydrate antigen (CA) 19–9 [16], carcinoembryonic antigen (CEA) and peanutagglutinin (PNA)-binding glycoproteins [17], human telomerase reverse transcriptase (hTert) [18], and matrix metalloproteinase-2 (MMP-2) [19]. More recent attempts to leverage circulating tumor cells and circulating free DNA have yielded similar results [20].

Different sources of pancreatic biomarkers have been evaluated, including blood serum and plasma, duodenal and pancreatic juice, and PDA tissue [21]. Various protein expression detection techniques have been developed, of which the mass spectrometry–based approach is perhaps the most promising. Comprehensive studies to catalog PDA specific proteins have been performed previously, including those by our group [21, 22, 23]. The clinical applicability of these studies was limited by the low concentrations of PDA specific proteins in peripheral blood. Current work is focused on developing and applying novel labeling techniques to improve sensitivity, multiplexing, and quantitative accuracy [24, 25].

A recent study reported proteomic and phosphoproteomic analysis of PDA tissue samples and normal tissue via a LC-MS/MS workflow. The investigators identified new candidate markers such as HIPK1 and MLCK from 2101 proteins identified [26]. They also demonstrated proteins involved in cell migration (Rho guanine nucleotide exchange factors and MRCKa) and formation of focal adhesion by phosphoproteomic analysis. They ascertained phosphorylation sites of known drug targets and suggested Fyn, ERK2, AKT1, and HDAC are potential targets for PDA treatment.

Humphrey et al. reported phosphotyrosine profiling of ATCC PDA cell lines and PDX cell lines they established by immunoaffinity-coupled high-resolution mass spectrometry [27]. They revealed three subtypes of ATCC cell lines, which are associated with cell-cell adhesion and epithelial-mesenchymal transition, mRNA metabolism, and receptor tyrosine kinase (RTK) signaling, respectively. One subtype of PDX cell lines is associated with RTK signaling and showed sensitivity to EGFR inhibitor, erlotinib. These results suggest that a phosphosignature may provide a predictive biomarker for response to targeted therapies.

Metabolomics

Targeting cancer metabolism requires personalized diagnostics for clinical success. Daemena et al. [28] identified three highly distinct metabolic subtypes through broad metabolite profiling of 38 PDA cell lines. One subtype was defined by reduced proliferative capacity, whereas the other two subtypes (glycolytic and lipogenic)

showed distinct metabolite levels associated with glycolysis, lipogenesis, and redox pathways, which were confirmed transcriptionally. The glycolytic and lipogenic subtypes showed striking differences in use of glucose and glutamine and showed differential sensitivity to inhibitors of aerobic glycolysis, glutaminolysis, lipid synthesis, and redox balance. In PDA clinical samples, the lipogenic subtype is associated with the Collisson classical subtype, whereas the glycolytic subtype is associated with the Collisson QM-PDA subtype. These findings suggest the utility of broad metabolite profiling to predict sensitivity of tumors to a variety of metabolic inhibitors.

Metabolism in Pancreatic Cancer: Clues from Metabolomics

PDA patients demonstrate many metabolic alterations including signs of muscle wasting, cachexia, fatigue, and changes in lipid and glucose metabolism. These changes cause alterations in levels and distributions of metabolites and recent technological advances have allowed for metabolomic profiling of a variety of relevant biological samples such as serum, tissue, and urine, with the potential for impacting diagnosis, prognosis and therapy. Detecting metabolic markers have been of intense focus in PDA. Many screens have been performed and these studies point to an important role of several metabolites and metabolic pathways.

It is generally understood that the development of tumors requires not only the ability to proliferate uncontrollably but also altered metabolic programs to sustain this rapid expansion. While there are changes common to multiple cancer types such as upregulated glucose uptake and lactate production, known as the Warburg effect, the metabolic profiles of individual tumors and tumors at different stages of development also possess unique features due to the heterogeneous nature of cancers. PDA tumors take up increased amounts of glucose to fuel biosynthetic processes, display elevated glutaminolysis to maintain redox balance, and scavenge fatty acids as well as amino acids from extracellular space to synthesize macromolecules such as lipids and proteins. These metabolic adaptations are the results of oncogenic signaling active in PDA and tumor microenvironment modulation, which collectively meet the cell's demand to accumulate biomass and proliferate.

Transcriptomic analysis leveraging a doxycycline inducible $Kras^{G12D}$ expressing genetically engineered mouse model (GEMM) and targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS) metabolomics revealed that $Kras^{G12D}$ is essential for glucose utilization through stimulation of glucose uptake and channeling of glucose intermediates into the hexosamine biosynthesis pathway for protein glycosylation and pentose phosphate pathways (PPP) for ribose production [29]. This functional validation of several $Kras^{G12D}$-regulated metabolic enzymes provides candidate therapeutic targets and associated biomarkers for the PDA oncogenic signature.

Kottakis et al. [30] provides evidence for a broader role of metabolic and epigenetic crosstalk in cancer pathogenesis, revealing that LKB1 mutant PDA cells have a marked dependency on pathways linking glycolysis, serine metabolism,

and DNA methylation. Their study provides evidence that coupled metabolic and epigenetic states have a more general role in cancer pathogenesis and suggest that LKB1 status is a genetic marker for DNA methyltransferase inhibitor responsiveness.

Recently, studies have focused on communication between tumor and stromal cells, which support tumor cell survival, growth, and proliferation. Notably, this crosstalk includes release of metabolites. Many studies have focused on the role of stromal cells as nutrient suppliers for PDA. Macropinocytosis-mediated internalization of extracellular proteins and their subsequent intracellular degradation was demonstrated as a mechanism for amino acid supply in Ras-transformed cancer cells. These findings suggest the inhibition of macropinocytosis as a promising strategy for therapeutic targeting in a subset of cancers.

Zhao et al. [31] show that fibroblasts smuggle essential nutrients to cancer cells via exosomes, and disable oxygen-based energy production in cancer cells. Oxygen-based energy release was dramatically reduced in the exosome-absorbing cells, and glucose-based energy release increased. They found that contents of the exosomes contain proteins, fatty acids, and other important molecules, which are used by PDA to proliferate. These findings suggest that preventing exosomes from smuggling resources to starving cancer cells might be an effective strategy to treat cancers. Stroma-tumor crosstalk remains under investigations, and this phenomenon reinforces the complexity of PDA. These studies provide new hints regarding the origin of metabolites and approaches to deprive tumors of their benefits.

RNA-sequencing of the PSC transcriptome revealed that, during activation, PSCs decrease expression of genes implicated in lipid storage and lipid metabolism and also increased expression of genes with tumor-supporting potential including cytokines, growth factors, ECM components, and signaling molecules such as Wnt. The transcriptomes of PSCs isolated from patients with PDA identified a PSC "cancer signature" [32]. These analyses also revealed that PSCs express high levels of the vitamin D receptor (VDR), which is maintained in the cancer-associated PSCs. Transcriptome analysis of preactivated and activated PSCs grown in the presence or absence of VDR ligand showed that the vitamin D receptor (VDR) acts as a master genomic suppressor of the PSC activation state. VDR ligand reduces fibrosis and inflammation in a murine pancreatitis model and enhances the efficacy of a coadministered chemotoxic agent. These results highlight a potentially widely applicable strategy to modulate stroma-associated pathologies including inflammation, fibrosis, and cancer.

To identify the marker for early diagnosis of PDAC, a number of studies have been performed in serum and, tissue and urine. In a study using gas chromatography mass spectrometry (GC/MS) on serum samples from patients with pancreatic cancer, Kobayashi et al. [33] investigated a diagnostic model based on four serum metabolites (xylitol, 1;5-anhydro-d-glucitol, histidine, and inositol) and found the profile to outperform both CA 19–9 and CEA for diagnosis.

Recently, Mayers et al. [34] reported that branched-chain amino acid (BCAA) serum levels are elevated 2–5 years before the onset of carcinogenesis in PDA, suggesting that BCAA elevation is an independent risk factor for PDA. Metabolic

changes alter systemic amino acid profiles together with changes in plasma BCAA concentrations in the precancerous phase or extremely early stages of PDA. However, BCAA levels return to normal levels within the 2 years before confirmation of cancer. In addition, the results of a mouse study indicated that the period of BCAA elevation was bell-shaped and only temporary. Fukutake et al. [35] indicated novel plasma free amino acids (PFAA) profiles from a large cohort of PDA patients. Concentrations of 19 PFAAs were measured by liquid chromatography–mass spectrometry. Plasma serine concentrations were especially elevated, while tryptophan and histidine concentrations were diminished in PDA patients compared with healthy control subjects. The PFAA profiles of PDA patients with stage 0–IIB disease, the resectable stage subgroup, were similar to those of all other PDA patients. This study identified characteristics of PDA phases, and the PFAA index is a promising biomarker for screening and diagnosis of PDA.

Zhang et al. found specific alterations in free fatty acid (FFA) metabolites, which were decreased in cancer patients [36]. Alterations in the lipid metabolism network included key lipolytic enzymes. Gene expression of these lipases was significantly decreased in pancreatic tumors as compared with nontumor tissues, leading to a reduction in FFA. These results may open new therapeutic options for targeting PDA.

Urinary metabolomics was explored using nuclear magnetic resonance (NMR) spectroscopy to investigate metabolomics profiles in the urine of PDA patients. A distinct urinary metabolomics signature was found in urine of patients with newly diagnosed PDA [37], which reliably could separate patients with PDA and controls with benign disease. Of particular interest was the finding that the increased urinary metabolomic profile decreased after surgical R0 resection.

While metabolomics studies using different technology platforms and samples from various tissue types can provide further insight into cancer biology, the current challenge with these results is confirming validity and reproducibility. Markers and panels appear to change across studies and technological platforms, thus making it difficult to find any one panel with a superior diagnostic, predictive, or prognostic value over the other. Metabolomic profiles of PDA patients have been reported in several previous studies, among which, several amino acid profiles were similar, although there were some obvious discrepancies. First, previous studies included relatively small numbers of subjects compared with the recent studies, which included the largest number of subjects to date. Second, differences may have occurred because of variations in sample preparation conditions and analytical methods. Third, metabolite profiles exhibit diurnal fluctuations and are largely dependent on recent meals. Furthermore, leaving collected blood samples at room temperature is known to alter plasma amino acid concentrations. Furthermore, genetic, racial, and geographical elements may also be factors impacting metabolic profiles, all issues which should be clarified in future research.

Tumors are often highly heterogeneous, with distinct areas dependent on different signaling pathways. Tumor cells adapt and reprogram their metabolism to cope with different environmental conditions. All this makes metabolomic mapping quite difficult. With the hypoxic versus normoxic mosaic, PDA perfectly reflects the

idea that different metabolic environments may be found within a single tumor mass, an area worthy of further study. As with other fields of study, tumor metabolism likely results from disturbances in several pathways and will require more sophisticated approaches going forward.

High-Risk Patients

Up to 10% of PDA occur in families with at least two affected first-degree relatives and these are designated familial pancreatic cancers (FPC). FPC is associated with a 2.3- to 32-fold increased risk of PDA development.

The International Cancer of the Patients Screening (CAPS) Consortium has recently reported a suggested guideline for screening, surveillance, and management of high-risk individuals with an inherited predisposition to PDA [38]. A consensus for a screening program to detect and treat T1N0M0 margin-negative PC and high grade dysplastic precursor lesions (pancreatic intraepithelial neoplasia and intraductal papillary mucinous neoplasm) was reached that the following groups should be offered screening (only to individuals who are surgical candidate): (1) first-degree relatives (FDRs) of the cancer patients from a familial pancreatic cancer cohort with at least two affected (FDRs); (2) patients with Peutz-Jeghers syndrome; and (3) p16, BRCA2 and hereditary nonpolyposis colorectal cancer mutation carriers with at least a single affected FDR. The initial screening should include EUS and/or MRI. However, consensus was not reached on the beginning and the end age of screening/surveillance and the interval of the examination. Their conclusions also included requirements for further studies, and the clinical management should occur at high-volume centers with multidisciplinary teams.

Recent advances in sequencing technology revealed PALB2 and ATM as FPC susceptibility genes, together explaining 3% to 5% of FPC cases. A further 8% to 15% of FPC patients have been reported to harbor other susceptibility genes, including BRCA1, BRCA2, CDKN2A, MLH1, MSH2, MSH6, PMS2, PRSS1, STK11, and TP53. Recent whole genome sequencing demonstrated deleterious variants in the candidate genes BUB1B, CPA1, FANCC, and FANCG as more frequent in FPC patients, many of which are associated with DNA repair or chromosomal stability. CPA1 gene variants have been shown to predispose to chronic pancreatitis, which is strongly associated with an increased risk of PDAC [39].

For FPC patients harboring BRCA1, BRCA2, or PALB2, targeting DNA repair with poly (ADP-ribose) polymerase 1 (PARP-1) inhibitors, platinum compounds, or mitomycin C showed therapeutic benefits [5].

Precision Medicine Clinical Trial

Although we have made great progress in understanding of PDA biology, translating these advances to effective, precision medicine remains a daunting challenge. Both the promise and challenge are illustrated in the IMPaCT (Individualized Molecular

Pancreatic Cancer Therapy) trial [6]. In this study, HER2 amplification, KRAS wild-type, and mutations in DNA damage repair pathways (BRCA1, BRCA2, PALB2, ATM) were screened in 76 samples derived from 93 patients.

In this trial, some challenges are illustrated. Of the 22 eligible patients identified for targeted therapy, none were able to receive treatment on protocol because of declining performance status or death. Median time from consent to molecular targeted analysis was 21.5 days. Delays occurred at external testing facilities ($n = 6$) and the requirement for a repeat biopsy ($n = 1$). These delays resulting from molecular analysis before treatment initiation are critical in PDA because of the rapid progression of this disease. Von Hoff and colleagues [40] showed that 17.9% (19/106) of participants were unable to be treated according to molecular analyses in a separate molecularly guided study due to worsening physical condition or progression of disease.

Allowing treatment to commence during analysis has not overcome the time lag and perhaps using molecular analysis performed during first-line therapy to guide second-line therapy may be a more practical approach. Randomization in certain studies can also be a deterrent to patient participation.

A paucity of material for molecular analysis remains a major problem. While FNA samples are mainly used for diagnostic material for metastatic PDA patients, the material that remains for molecular analysis is frequently unsuitable. These samples yield low amounts of DNA which is of poor quality for sequencing. Furthermore, as PDA tissue is of low cellularity, limiting eligibility to biopsy samples with cellularity as high as the cancer genome atlas (>60%) would exclude many patients.

Using surrogate biospecimens to perform molecular analysis is a promising approach to overcome some of these obstacles, for example, circulating tumor cells [41] or cell-free DNA. Innovative in vitro approaches, such as expansion of small numbers of tumors cells in three-dimensional organoid culture, can generate adequate numbers of tumor cells, for molecular analysis. Significant efforts are under way to explore these approaches for clinical applicability. Cancer knowledge networks also need to be built to store the results of molecular analysis and medical data of patients, which can then be shared in comprehensive ways among scientists, health care workers, and patients.

Preclinical Models

Cancer Cell Lines

Cancer cell lines have been important tools for drug development. Studies from The Cancer Genome Atlas (TCGA) and the International Cancer Genome Consortium (ICGC) have established comprehensive catalogs of cancer genes involved in tumorigenesis.

Large-scale drug sensitivity screens in cancer cell lines have been performed to identify potential active drugs. The National Cancer Institute Developmental Therapeutics Program has studied and developed more than 100,000 chemical

compounds using 60 human cancer cell lines (NCI-60) since 1990, and this panel of cell lines continue to be used for in vitro drug screening and development.

Two recent projects, the Genomics of Drug Sensitivity in Cancer (GDSC) and the Cancer Cell Line Encyclopedia (CCLE) have evaluated genetic correlations of drug sensitivity. GDSC assembled 639 human tumor cell lines and 130 drugs for screening. CCLE described gene expression, chromosomal copy number, and massively parallel sequencing data from 947 human cancer cell lines and the drug response of 24 compounds across 479 cell lines.

PDA cell lines continue to play an important role in studying biology and drug development. Phenotype and genotype of many of these cell lines are well established. Cell lines are homogeneous, grow rapidly in culture, and are easy to study.

Collison et al. [13] evaluated 19 human and 15 mouse PDA cell lines and showed these cell lines do not cover all subtypes of PDA found in patients. They compared their data sets from 27 human microdissected tumors to human and mouse cell lines. Cell lines most closely modeled either classical or QM-PDA subtypes. Classical type was more dependent on Kras than QM-PDA as determined by RNAi. Kras targeted therapy, therefore, may be effective against classical type tumors [42]. QM is more sensitive to gemcitabine than classical and classical is more sensitive to erlotinib than QM.

Generating cancer cell lines results in certain alterations in biologic properties, such as genetic alteration, alteration in growth and invasion properties, and loss of specific cell populations. In addition, cell lines are usually established only from more aggressive tumors and hence are not representative of complex tumor heterogeneity.

Garnett et al. screened 639 human cancer cell lines, representing most tissue types and a wide range of genetic diversity of human cancers to uncover new biomarkers of sensitivity and resistance to cancer therapeutics, using 130 drugs under clinical and preclinical investigation. Cell lines were subjected to sequencing of the full coding exons of 64 commonly mutated cancer genes, copy number analysis, and expression profile. In addition to well-established targeted therapies, such as BCR-ABL-positive CML, BRAF-mutant melanoma, and EGFR-mutant lung cancer, they showed sensitivity of EWS-FLI1-positive Ewing's sarcoma cell lines to PARP-inhibitors [43].

Iorio et al. [44] analyzed somatic mutations, copy number alterations, and hypermethylation across a total of 11,289 tumors from 29 tissue types and reported how these alterations can be mapped onto 1001 human cancer cell lines and correlated with sensitivity to 265 drugs. They demonstrated that a sufficiently large panel of cancer cell lines recapitulates oncogenic alterations in primary tumors. However, many genetic alterations occurring at low to moderate frequencies (2–5%) are only represented by a single cell line or not at all, and coverage by cancer type is variable. They analyzed the most predictive data types in pan-cancer and cancer-specific analyses. In cancer specific analyses, genomic features generated the most predictive models, while in the pan-cancer analyses, baseline gene expression data was less informative.

Cell Line Base Xenograft Model

Mouse models are the most experimentally tractable mammalian systems for advancing basic understanding of cancer biology. The xenograft mouse model has been widely used as a tool for preclinical drug screening. Human cancer cell lines can be transplanted either orthotopically or ectopically (usually subcutaneously) into immunocompromised mouse. T-cell deficient nude athymic, B and T lymphocytes deficient severe combined immunodeficient (SCID) and SCID on nonobese diabetic background (NOD/SCID) are commonly used host mice.

Among mouse models, the subcutaneous xenograft is a convenient and economical approach and allows for convenient tumor size assessment. Xenografts have facilitated analyzing the efficacy of compound testing, and most currently approved therapies have been preceded by xenograft testing. While xenograft screening in the earliest stages of drug development can be informative, the extensive screening by the NCI demonstrates a moderate predictive value for their xenograft models, and a poor correlation between the therapeutic efficacy in xenografts and in humans [45]. For PDA, a low correlation between in vitro testing data and clinical utility was also reported [46] .

Subcutaneous tumors are a homogeneous mass with limited stromal infiltration and rarely metastasize. Orthotopic transplantation, where cancer cells are transplanted into the relevant tissue of origin, is better than subcutaneous transplantation for modeling tumor stromal interactions. As metastatic models, cancer cells can be injected intravenously, commonly in the tail vein to model lung metastases, or intraventricularly to model systemic metastases. To model liver metastases, cancer cells are injected into the portal vein or spleen. These transplantation systems can be adapted to many different cancer types.

There are also several shortcomings for xenograft mouse models. Host (SCID and nude) mice are immune deficient and not useful for testing of immunomodulatory agents. In addition, in these systems the immunodeficient state of the mouse results in the failure to completely recapitulate the complex tumor-stromal interaction and the impact on drug response of the tumor microenvironment. These are important considerations particularly in PDA, which is characterized by an abundant stromal reaction and unique heterogeneity. Xenograft studies typically use only a few human tumor cell lines, the oncogenomic profiles of which represent only isolated combinations of the wide spectrum of genetic and epigenetic mutations that are resident in the tumors found in human patients. The reliance on small numbers of homogeneous cell lines is a fundamental weakness.

Genetically Engineered Mouse Model

By using pancreas-specific conditional activation or knockout of clinically relevant PDA-related genes and signaling pathways, genetically engineered mouse models

(GEMM) of PDA have been described and are now a well-established tool. Histologically, PDA GEMMs generally develop differentiated ductal adenocarcinoma with abundant stromal components including a robust desmoplastic reaction. Some GEMMs develop sarcomatoid or undifferentiated tumors, which are rare in human pancreatic cancer. With regard to TGF-beta signaling, SMAD4 gene mutation or deletion is frequently observed in human PDA tumors; however, mice engineered with pancreas specific Kras activation together with Smad4 knockout were reported to develop cystic tumors of the pancreas, a precancerous lesion distinct from PanINs, intraductal papillary mucinous neoplasms, or mucinous cystic neoplasms [47].

An excellent review of a large number of mouse models was performed, and describes several differences between the pathology identified in GEMMs and that seen in human tumors [48]. First, human PDA tends to be moderate or poorly differentiated, whereas many of the GEMMs produced anaplastic carcinomas. Second, most neoplasms in humans show a single direction of differentiation, whereas multilineage differentiation, including acinar differentiation, was often seen in GEMMs. Third, pancreatic intraepithelial neoplasia in humans often, although not always, occurs in the pancreatic duct. By contrast, many of the duct lesions in GEMMs arose in the background of diffuse acinar-ductal metaplasia. Fourth, most human pancreatic carcinomas are solitary, whereas multifocality, not surprisingly, is commonly seen in GEMMs. Finally, intense desmoplasia is a characteristic feature of invasive ductal adenocarcinoma in humans. By contrast, little desmoplasia is seen in some GEMM carcinomas. Each of these models has its own unique strengths and weaknesses in advancing our understanding of pancreatic neoplasia, to identify target-specific biomarkers to assess drug action and discover resistance mechanisms.

PDA GEMMs have been utilized to make important discoveries. One of the earliest studies described how PDA GEMMs appear to recapitulate the tumor microenvironment better than xenograft tumor models. The GEMM also recapitulated chemotherapy resistance, similar to what is seen in the human disease [49]. One of the most commonly used GEMMs for evaluating preclinical therapeutic agents is the PDX-1-Cre; LSL-$Kras^{G12D}$; LSL-$p53^{R172/-}$ (KPC) model [50]. The KPC model recapitulates the clinical features of PDA including hemorrhagic ascites and cachexia. This model also demonstrates metastases to liver, lung, peritoneum, and lymph nodes and a short median survival of approximately 5 months. Histopathologically, tumors generally demonstrate ductal adenocarcinoma with dense stromal desmoplasia; however, sarcomatoid and anaplastic tumors do also occur. Unlike xenograft models using immunocompromised mouse, GEMMs have an intact immune system and stromal reaction. An intact tumor microenvironment was important for the preclinical study of PEGPH20 [51], a PEGylated human recombinant PH20 hyaluronidase. The glycosaminoglycan hyaluronan (HA) is abundant in PDA stroma and transduces signaling through CD44 to regulate receptor tyrosine kinases and small GTPase activity which play important roles in angiogenesis, epithelial-mesenchymal transition, and chemoresistance [52]. PEGPH20 treatment increases intratumoral delivery of chemotherapeutic agents by digesting HA. These preclinical studies have prompted further clinical development of PEGPH20, which is currently in randomized phase III testing for the treatment of advanced PDA

(NCT02715804). Hedgehog pathway inhibition was first reported to inhibit the stromal component in KPC mice, which increased the delivery of gemcitabine to tumors and improved survival in combination with gemcitabine [49]. Unfortunately, in a randomized phase II clinical trial, the hedgehog pathway inhibitor IPI-926 in combination with gemcitabine was ineffective. Using a separate GEMM, Rhim et al. demonstrated that prolonged hedgehog inhibition as a monotherapy led to more aggressive tumor behavior [53]. These results suggest GEMM models are a useful tool to evaluate the efficacy of drugs targeting tumor microenvironment and mechanism of efficacy of chemotherapeutic agents. GEMM models also play an important role in evaluating immune modulating agents. Feig and colleagues reported that KPC models do not respond to antagonism of the immune checkpoints anti-cytotoxic T lymphocyte-associated protein 4 (α-CTLA-4) and α-programmed cell death 1 ligand 1 (α-PD-L1), as is seen in human clinical trials. However, the depletion of cancer-associated fibroblast enabled control of tumor growth using these inhibitors. Treatment with a CXCL12 receptor inhibitor resulted in T cell accumulation in tumors and potentiated anticancer effects of α-PD-L1 [54]. GEMMs can be used to understand the disease biology and drug development, particularly focused on tumor microenvironment and immune response.

It is evident that an understanding of genetic events and signaling pathways is crucial for the development of effective targeted therapies in PDA. GEMMs will continue to play a significant role in the crucial first step of drug discovery and target validation. Pdx1-Cre; LSL-$Kras^{G12D}$; $Pten^{flox/flox}$ mouse model which demonstrates elevated mTOR (mammalian target of rapamycin) signaling showed response to mTOR inhibitor [55]. In clinical trial, mTOR inhibitor did not show the efficacy for unselected pancreatic cancer patients. However, patients with mutations in mTOR pathway showed efficacy for mTOR inhibitor [55]. A Ptf1a-Cre; LSL-$Kras^{G12D}$; $Tgfbr2^{flox/flox}$ mouse model was used to assess the efficacy of the EGFR inhibitor erlotinib in combination with gemcitabine [56]. Systematic studies using 2D cancer cells of cancer genomes and drug efficacy implied the efficacy of EGFR/ERBB2 inhibitors against cancer cells with Smad4 mutation [44].

Recent whole genome sequencing, exome sequencing and RNA sequencing studies revealed some characteristics of PDA, but these subtypes are not predictive for drug sensitivity. GEMMs recapitulate many of the features of human PDA. GEMMs can be useful to evaluate drug response against PDA patients with specific genetic backgrounds. With regard to the discovery of specific biomarkers in cancer patients, it is necessary to collect large numbers of specimens because of interindividual variability, which makes the discovery of biomarkers difficult. However, the use of a GEMM, designed to develop the desired cancer with a predicted latency could allow for identification of candidate biomarkers, which can then be validated in human clinical samples.

By using tetracycline-regulated and CRE-inducible alleles, the timing, duration and tissue compartment of gene expression or inactivation can be further controlled. An alternative method for generating GEMMs uses the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated proteins) gene-editing system. Chiou and colleague reported CRISPR-mediated targeting of liver

kinase B1 (LKB1) in combination with Kras expression [57]. In this study, they also reported in vivo gene editing by retrograde injection of adenoviral-Cre and lentiviral-Cre into the pancreas of LSL-KRas^{G12D}; p53$^{flox/flox}$ mice.

GEMMs are an important tool for studying biology and drug development. GEMMs are customizable to perturb any number of genetic alterations, which will hopefully continue to lead to more effective therapies.

Patient Avatars

Patient-Derived Xenograft

For a number of reasons previously discussed, the establishment of cell lines is not an effective strategy for personalized medicine. The principal limitation of conventional 2D cell line–based xenograft models is their poor predictive value with regard to clinical outcome [58]. Generally, PDX models have been reported to retain the principal characteristics of donor tumors both histologically and biologically. An analysis of genetic profiles show good concordance between primary tumors and the models derived from them, except discordance in genes involved in the stromal compartment and immune function, which is due to the replacement of the human stroma by murine elements. Although the gene expression profile of PDX models is similar to the original tumor, cell lines developed from the same specimen demonstrate a different expression profile that is not restored by in vivo subcutaneous propagation in mice in SCLC. In PDA models, similar results have been observed in which the frequency of mutations in genes such as TP53 or RAS closely mirrors the frequency of these mutations in human samples [59, 60].

PDX models are an attractive preclinical tool to improve drug screening and development. PDX models are expected to faithfully model the human patients from whom the tumor is derived, both with regards to cancer biology and response to treatments. Personalized PDX models have been studied as a tool for testing candidate regimens which may be effective for treating the patient's own tumor [61]. Evaluating the relationship of drug response with genetic information could lead to the discovery of new biomarkers of drug efficacy. These results suggest that PDX models hold promise for precision medicine in PDA.

One study found a good correlation between response in patient derived PDX and clinical response to gemcitabine in PDA patients [62]. Drug response of PDX models has been reported to be stably maintained across generations (up to 10 passages) [59].

Hidalgo et al. found in a pilot study that treatment of PDA patients with drugs selected according to preclinical PDX drug screening was predictive of tumor response, which suggests that response in PDX models correlates with clinical outcome [63]. This work showed that the combination of nab-paclitaxel and gemcitabine is effective in PDX models of PDA, which correlated with the clinical efficacy of this combination. This regimen has subsequently been demonstrated to provide a survival benefit for patients with advanced PDA in a randomized phase III

study. Likewise, failure to exert antitumor efficacy in PDX models correlates with negative clinical results. This is illustrated in PDAC for agents such as the SRC inhibitor saracatinib and the mTOR inhibitor sirolimus, for which lack of efficacy in unselected PDX preclinical studies predicted failure of the same strategy in the clinic [61]. Based on these data, PDX models have now become an integral part of the preclinical screening of new anticancer agents.

The concordance between PDX models and human trials with regard to biomarkers of drug susceptibility and drug resistance is an important finding. In PDA, PDX studies with gemcitabine identified expression of the gemcitabine-activating enzyme deoxycytidine kinase as a predictor of drug efficacy [59, 64]. Likewise, PDX models have been used to identify metabolic as well as imaging biomarkers. PDX models are also versatile tools for simulating resistance when exposed to treatment strategies used in the clinical setting and to study strategies for overcome resistance.

In most patients, derivation of a personalized PDX for guiding therapy is not feasible for a combination of reasons such as failure of the tumor to engraft, lack of effective agents, and length of time required for a complete study [62, 63]. For patients whose tumors do not take in mice or those who require a long time to be established and characterized, an alternative to a personalized PDX strategy could be to determine treatment choices based on drug responses in a similar, established PDX. Biopsies of primary tumors or metastases would be molecularly characterized and compared with available PDX collections from the same pathology, for which responses to chemotherapies and targeted agents have been previously determined.

PDX models generally rely on surgical specimens, which provide large quantities of tumor tissue. As most PDA patients are inoperable, it is more useful to generate PDX from smaller samples, such as fine-needle aspiration for personalized therapy. Four to eight months are required to generate PDX models for preclinical treatment study. The success rate of engraftment is about 60% and it is important to establish the best engraftment methods according to the phenotype of cancer. Human cancer stroma included in the cancer specimens are replaced rapidly by mouse stromal cells including fibroblasts, inflammatory cells, blood vessels, and immune cells. PDX models require an immunocompromised mouse host which limits the ability to evaluate immune modulators, such as vaccines, anti-PD-1, and anti-CD40 antibodies.

PDX models may also be used as part of co-clinical trials. In co-clinical trials, a personalized PDX model is developed from a patient enrolled in a clinical trial and treated with the same experimental agents to emulate clinical response by using appropriate endpoints such as response rate or tumor growth delay. The availability of a larger collection of models extensively characterized at the histologic, molecular, and genomic level would enable these larger screens. Biologic and genetic comparisons between sensitive and resistant models can be explored for the prioritization of biomarkers for inclusion in clinical studies.

This strategy permits the assessment of drug response simultaneously in the patient and mouse model, providing an interesting platform to investigate biomarkers of susceptibility and resistance, as well as interrogation of novel combination strategies to overcome emergent resistance pathways. Novel approaches, such

as short-term primary cultures or organoids, are being developed and are expected to be used for preclinical screening studies.

Organoid: A Promising New Model

New and innovative culture approaches have been developed which address several obstacles to studying and treating PDA. As previously discussed, samples for genetic screening are frequently unsuitable, of low cellularity, yield low quantities and poor quality DNA for sequencing. 2D cell lines established from human PDA samples are useful; however, the process of cell line establishment results in clonal loss, therefore cell lines do not accurately reflect tumor heterogeneity.

Loss of tumor heterogeneity is a similar weakness of 2D cell line–based xenografts. While studies of PDX models have demonstrated the presence of dense desmoplastic stroma, maintenance of tumor heterogeneity, and good correlation between drug response and human clinical response, transplant success rates are biased towards more aggressive tumors and require a large piece of tumor tissue. PDX models require 4 to 8 months before drug screening can be performed. GEMMs recapitulate the stromal reaction, genetic mutations and progression from normal to PanIN to adenocarcinoma; however, GEMMs lack the genetic and cellular heterogeneity which can only be captured in the human disease.

New 3D culture techniques have been developed in the past decades, providing a new tool with the potential for addressing many of the issues described above.

The first description of this long-term culture system, termed organoids, was reported by Sato et al. [65]. Sato and colleagues used cells derived from the murine small intestine. Several key growth factors appear important for long-term organoid maintenance. For example, supplementation with Wnt ligand supports crypt proliferation, epidermal growth factor (EGF) supports intestinal proliferation, Noggin induces expansion of crypt numbers, inhibition of anoikis is necessary, and finally, laminin-rich Matrigel acts as an extracellular matrix and supports intestinal epithelial growth. At the same time, another long-term culture was established by Ootani et al. [66] for small and large intestine. Successively, long-term 3D culture methods were described for other organs such as stomach, liver, and mammary gland. In addition, long-term 3D culture system was described for malignant tumors derived from breast, colon, and prostate. More recently, normal pancreas and PDA organoid systems have been established.

Boj et al. [67] described an organoid culture system for both normal and neoplastic epithelial cells derived from both mice and humans. Pancreatic organoids are embedded in Matrigel, which contains essential components of a basement membrane. The culture media contains Wnt3a, Noggin, EGF, and R-spondin-1, which are key growth factors. For human organoid culture, FGF10, nicotinamide, A83–01, and prostaglandin E2 are additionally required. Pancreatic organoids can be passaged indefinitely except for human normal organoids, which generally can only be cultured for 20–25 passages. PDA organoids can be expanded from a minimal piece of tissue, such as from a fine needle aspiration (Fig. 1).

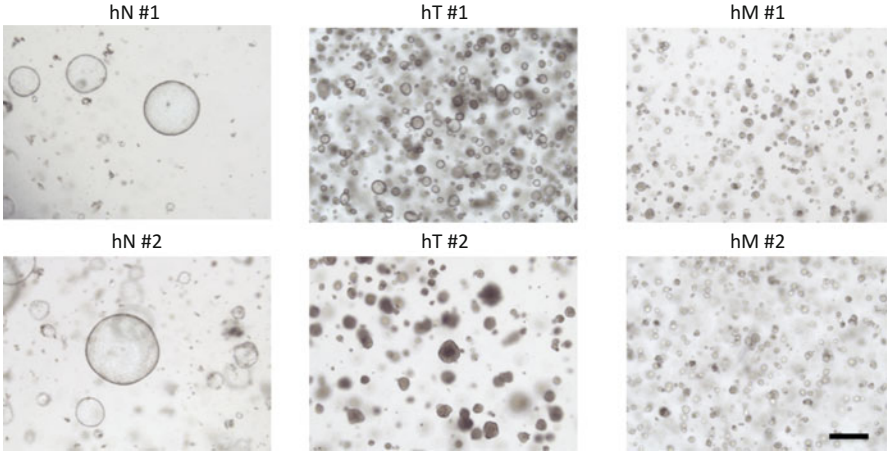

Fig. 1 Representative images of human organoid cultures established from normal tissues (hN), primary tumors (hT), and metastatic tumors (hM). Bar: 500 μm

Expansion of small amounts of tumor or normal tissue to large-scale organoid cultures allows for parallel precision medicine analysis including drug screening, genomic, transcriptomic, metabolomics, and proteomic analyses. Boj et al. performed gene expression analysis comparing mouse normal, PanIN, and tumor organoids and showed similar changes in gene expression patterns comparing mouse PanIN and tumor organoids to normal organoids, as seen with oncogenic Kras activation in KrasG12D mice. These analyses demonstrated the ability of the organoid system to characterize molecular alterations associated with PDA progression. Proteomic analysis of mouse normal, PanIN, and tumor organoids was also performed. Few protein expression changes were seen comparing mouse PanIN and tumor organoids, whereas many more changes were seen comparing mouse normal and PanIN organoids, or mouse normal and tumor organoids. Gene Set Enrichment Analysis (GSEA) of RNA sequencing and proteomic data comparing mouse PanIN to normal organoids revealed up regulated genes and proteins involved in glutathione metabolism and biological oxidations, consistent with previous studies. Similar to the PDX mouse model, organoid transplant mouse models are a promising tool for drug screening and studying biology. Using organoids for in vitro drug screening is possible a couple of months after samples are collected. Organoids can be reliably established from virtually every patient sample. Preliminary studies suggest maintenance of tumor heterogeneity even after several passages.

Interestingly, orthotopic transplantation of organoids develops a full spectrum of lesions associated with disease progression, including early PanIN and late PanIN, invasive ductal adenocarcinoma, and metastasis. This model is a promising tool to study the earliest stage of human cancer to understand fundamental biology and to identify biomarkers of early disease.

Hunag et al. generated pancreatic progenitor cells from pluripotent stem cells in 3D culture and induced differentiation of their organoid progenitor cells into

pancreatic exocrine cells which express ductal and acinar markers [68]. They adapted their culture condition for growing human PDA. Among 20 human pancreatic samples, they established 17 tumor organoid lines and showed similar morphological and cytological features to those of the primary tumors they were derived from after 16 days in 3D culture. They transplanted 50,000 cells subcutaneously and tumors grew within 4–7 weeks. Xenograft tumors demonstrated similar histoarchitecture to the primary tumor or origin and also maintained histological heterogeneity. They tested an EZH2 (enhancer of zeste homolog 2) inhibitor against human tumor organoids and suggested the usefulness of organoids as a platform for personalized drug testing, although they were not able to correlate organoid response to patient outcomes.

Walsh et al. established mouse and human organoids for drug testing and optical metabolic imaging (OMI) which probes the fluorescence intensity and lifetime of NAD(P)H and FAD [69]. After mechanical digestion, organoids are embedded in Matrigel and subjected to drug testing and optical metabolic imaging. This method does not allow for passage of organoids but can be useful as a tool to evaluate drug response for personalized medicine. They observed three distinctive morphologies of murine PDAC including spherical organoids (type 1), symmetric organoids (type 2), and fibroblasts. Type 1 and type 2 organoids are positive for epithelial markers. Type 1 organoids show the greatest OMI index and type 2 organoids showed the smallest OMI index. Optical redox index ratio of type 2 organoids was lower than that of type 1 organoids and fibroblast. Organoids were treated with a JAK2 inhibitor, MEK inhibitor, PI3K inhibitor, and combinations to evaluate drug-induced metabolic changes, which revealed heterogeneous metabolic responses among cell populations [69]. Human PDAC organoids demonstrated a broad spectrum of morphologies, which were difficult to classify into subtypes. They showed that the OMI index reduction was detected with gemcitabine treatment and gemcitabine with JAK2 inhibitor treatment.

Li et al. cultured organoids with both epithelial and mesenchymal components from embryonic pancreas using an air-liquid interface culture method with an inner collagen gel-containing transwell with direct air exposure. This system does not require exogenous factor supplementation [70].

Wetering et al. [71] reported the establishment of a “living biobank” from 20 colorectal cancer patients. They demonstrated that the organoid culture platform can be exploited for genomic and functional studies at the level of the individual patient for personalized treatment approach. Organoid technology may fill the gap between cancer genetics and patient trials, complement cell-line- and xenograft-based drug studies, and help to achieve an effective, personalized therapy approach.

Conclusion

Integrated genomic, epigenomic, and transcriptomic analyses are generating biological insights with potential therapeutic relevance in PDA. The recurrently mutated genes aggregate into core molecular pathways including KRAS, Wnt,

Notch, DNA damage repair, RNA processing, cell cycle regulation, TGF-β signaling, SWI-SNF, chromatin regulation, and axonal guidance. Genomic-based treatment has resulted in paradigm changing therapies for other cancers, dramatically improving survival and cures. However, this remains an unfulfilled promise in PDA due to apparently untargetable mutations, high resistance to available chemotherapeutic agents, and the difficulty of drug delivery through a rich stromal component. In addition, individual tumors have infrequently mutated genes, result in significant inter- and intratumoral heterogeneity. Due to this diversity, therapeutic development has been challenging. Familial pancreatic cancer patients harboring BRCA or PALB2 may have sensitivity to PARP-1 inhibitors, platinum compounds, or mitomycin C. In the IMPaCT (The Individualized Molecular Pancreatic Cancer Therapy) trial [6], HER2 amplification, KRAS wild-type, and mutations in DNA damage repair pathways (BRCA1, BRCA2, PALB2, ATM) were targeted for treatment. Personalized PDX models have the potential to identify effective drug therapies, however, with significant limitations, including a long lead-time and large amounts of tumor tissue for testing. The three-dimensional organoid culture platform can be exploited for genomic and functional studies at the level of the individual patient for personalized treatment approach. Organoid technology may fill the gap between cancer genetics and patient trials and allow personalized therapy design, although further studies to validate this approach are needed (Fig. 2). A combination of genome-based medicine and individualized model drug screening may prove to be the key tools needed for precision medicine for PDA (Table 1).

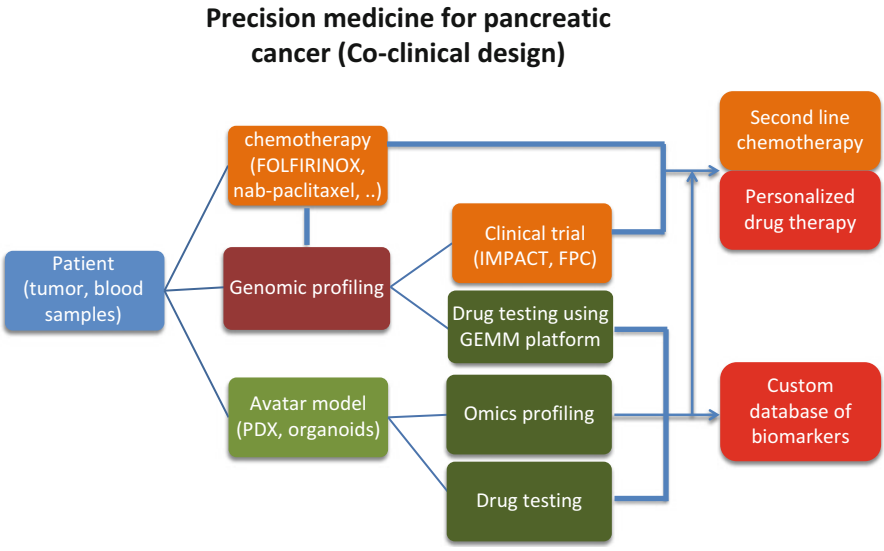

Fig. 2 The design of precision medicine

Table 1 Summary of main characteristics of different preclinical models

	2D cell lines	2D cell line–based xenograft	GEMMs	PDX	Organoids	Organoid-based transplant
Cost of maintenance	+++	++	+	+	+++	++
Success rate of initiation	+	++	+++	++	+++	++
Expansion	+++	+	++	+	+++	+
Genetic manipulation	+++	+	++	+	++	+
Tumor stromal interaction	–	+	+++	+	+++	+
High-throughput drug screens	+++				++	
Tumor heterogeneity	+	+	+	+++	++	++
Immune system	–	+	+++	+		+

Cross-References

- ▶ Approaching Pancreatic Cancer Phenotypes Via Metabolomics
- ▶ Chemotherapy for Advanced Pancreatic Cancer
- ▶ Clinical Decision-Making in Pancreatic Cancer
- ▶ Development of Novel Diagnostic Pancreatic Tumor Biomarkers
- ▶ Development of Novel Therapeutic Response Biomarkers
- ▶ Diagnostic Biomarkers
- ▶ Differential Therapy Based on Tumor Heterogeneity in Pancreatic Cancer
- ▶ Emerging Therapeutic Targets in Pancreatic Adenocarcinoma
- ▶ Epigenetic Pharmacology
- ▶ Familial Pancreatic Cancer
- ▶ Metabolism in Pancreatic Cancer
- ▶ Mouse Models of Pancreatic Exocrine Cancer
- ▶ Precision Medicine Based on Next-Generation Sequencing and Master Controllers
- ▶ Secondary Screening for Inherited Pancreatic Ductal Adenocarcinoma

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Epigenetic Pharmacology

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Abstract

Decades of research focused on the genetic basis for development of pancreatic ductal adenocarcinoma have yielded tremendous discoveries. Clues to increase our understanding of the underlying biology of disease, the time along which the disease develops, and the potential vulnerabilities of disease are being elucidated daily. Alongside this genetically driven paradigm, researchers have uncovered the phenomenon of dramatically altered protein expression in the absence of an associated gene mutation. Through a mechanism termed epigenetics, the transcription and translation of genes can be dramatically altered by a variety of mechanisms including DNA methylation and histone modification. The fundamental concepts of epigenetics and major molecular agents that participate in setting the epigenome are reviewed herein. For each mechanism, the pharmacologic agents available for current use and the research underlying their approval are discussed. The potential impact of epigenetic pharmacology in pancreatic cancer is discussed in turn, and future directions of current research efforts are outlined.

Keywords

Pancreatic ductal adenocarcinoma · Epigenetics · Epigenetic pharmacology · DNA methylation · Histone modification · DNA methyltransferase · DNA methyltransferase inhibitor · Histone deacetylase inhibitors

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related death in the United States [1]. With a mortality rate that approaches the incidence, the outcomes following diagnosis are dismal. There are many reasons that account for this statistic: advanced stage at presentation, aggressive underlying tumor biology, and relative inefficacy of standard therapies. It is the latter that often drives mortality. Whereas progress with systemic therapies has led to prolonged survival in many malignancies (including breast, colon, and gastrointestinal stromal tumors), cytotoxic chemotherapeutics have negligible benefit in survival after a diagnosis with PDAC. Research to associate genetic profiles with treatment response has also yielded disappointing findings. Alternative mechanisms of disease biology and treatment response are in active development.

Introduction to Epigenetics

The central dogma of molecular biology posits that genetic information coded in DNA is transcribed into RNA and translated into protein. Protein then functions in

such a way to ensure that the phenotype expressed by a cell accurately reflects the cell's underlying genotype. The recognition of this oversimplification occurred in parallel with the discovery of the genome itself, as it was clear that cells containing the same genome expressed widely disparate phenotypes (e.g., note the differences between a hepatocyte and a melanocyte). Even today the forces driving the development of a particular phenotype remain incompletely understood; however, the mechanisms used by cells to establish these differences are increasingly being unraveled. Examples of these mechanisms include variable transcription from the DNA, regulation of RNA translation, and regulation of protein expression.

With transcription alone, it is important to remember that the DNA is not always freely available for copy into RNA. At baseline, portions of the genetic code are twisted and wrapped around alkaline proteins, termed histones [2]. These histones, together with the DNA and other nuclear proteins, form tightly spiraled nuclear structures, called nucleosomes, which can promote or restrict access to DNA by the translational machinery of a cell. Further, even when not tightly bound to histones, specific residues of the DNA can be shrouded behind methyl groups (CH_3) prohibiting their transcription (as discussed later in this chapter). In cases such as these, when DNA is wrapped into tight complexes or covered by methylation, the expression of genes can be significantly altered.

Epigenetics is the term used to characterize the mechanisms of variable gene expression leading to disparate cellular phenotypes due to changes in a chromosome, without changes in the underlying sequence of DNA [2]. Though chromatin structure and nucleotide methylation are commonly cited examples of epigenetic variability, there are many other potential cellular processes with the capacity to exert epigenetic influence on a cell. These include changes in RNA or microRNA profiles that bind and augment the structure or function of histones, changes in nuclear protein composition that may fundamentally alter the microarchitecture between histones, or metabolic changes that can modify epigenetic protein binding or affinity. Commonly, these global changes within a cell can result in histone modifications by way of acetylation, ubiquitylation, sumoylation, and methylation.

Epigenetic changes are believed to be heritable with a potential impact just as great as germ line mutations in the DNA sequence [2]. Even after gestation and throughout the duration of life, epigenetic events are durable and persist from one cell division to the next. Importantly, however, the epigenetic profile of a cell (i.e., the epigenome) can be dynamic, reacting to environmental signals and allowing for changes to accumulate. At times this is likely a protective mechanism, helping to guide cellular fate during embryogenesis and adult cell renewal [2]. In stark contrast, alongside genetic mutations that drive malignancy, there are changes to the epigenome that appear to be early events in cancer tumorigenesis. In this chapter, the rationale for broadening research into novel therapeutics based on recent epigenetic studies is highlighted. The current mechanisms of epigenetic control are detailed as a framework from which to discuss potential pharmacologic therapies. Finally, ongoing studies and anticipated future work are highlighted.

Epigenetics: Definitions and Basic Mechanisms

Despite an increasing understanding of the DNA mutational landscape driving cancer, the progress made in developing therapeutics has been disappointing. While there are many reasons for this, one prominent hypothesis rests on the vast machinery that regulates the expression of the cell's genotype. In a simplified model, each gene encoded by DNA would be transcribed into RNA, be translated into protein, and then contribute to a cell's fate through the protein-protein interactions detailed in biochemical and molecular biologic texts. In reality however, there are dramatic differences in the ultimate production of protein encoded from one gene to the next on the chromosome. Some of this variability is due to regulation of RNA translation or protein-level degradation. However, much of this variability is due to differences in the amount of DNA transcription that occurs at each gene location on the chromosome and is controlled by local factors. These local factors, that change the gene expression patterns in a cell, can be due to two nuclear phenomena in the epigenome. First, changes in gene expression can result from the nuclear protein interactions with DNA that form chromatin (the local arrangement or "micro-architecture" of the chromosomes). The resulting microarchitecture is sometimes referred to as the "histone code" [3]. Second, gene expression can be augmented by the direct methylation of DNA residues. Finally, microRNA and other noncoding RNA molecules can have profound effects on gene expression.

Chromatin Modification: Histone Modification, The "Histone Code"

The microarchitecture of chromosomes within the nucleus of a cell is dependent upon the relationship between the DNA and nuclear proteins (Fig. 1). In some cases, the DNA may be loosely splayed open in a bath of transcription factors and electrolyte solution, termed euchromatin. In other areas, the DNA is tightly bound to spherical nuclear proteins with the nucleotides shielded from view, termed heterochromatin. It is this relationship, between the DNA and alkaline-rich proteins called histones, which is the major determinant of chromosome shape and function. Around each histone core, approximately 160 base pairs of DNA are wrapped. Together this complex is called the nucleosome. Each nucleosome may also bind tightly to a neighbor or be distanced from each other and stand apart at length. The positioning of nucleosomes in relation to their neighbors helps to form macrostructures termed chromatin. Chemical modifications to the core of histone proteins are the major determinants of chromatin arrangement (Fig. 1) [4].

Over the past two decades, major strides have been made to increase understanding of the mechanisms controlling the epigenome. Expression of genes along any length of DNA is dependent upon the arrangement of the chromatin and nucleosomes. As transcription start sites are wrapped tightly, the transcription machinery cannot intercalate with the DNA to facilitate gene expression. In contrast, as the start sites in the DNA move away from the nucleosome, they become more available for transcription. Nuclear proteins that function within intricate complexes control these

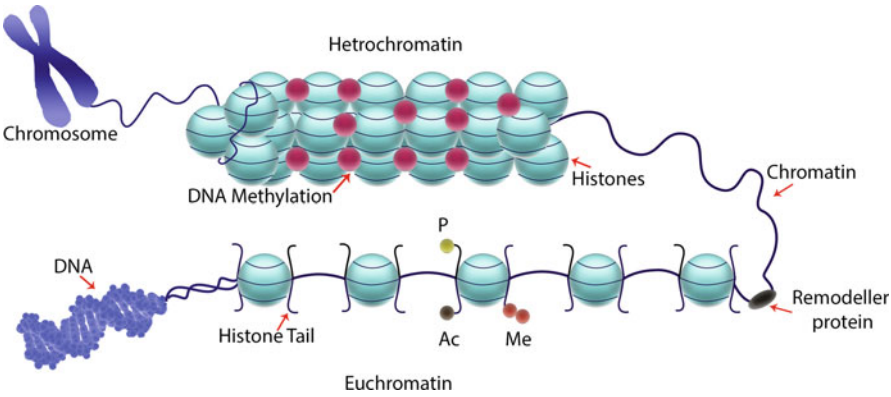

Fig. 1 The architecture of epigenomic landscape. The chromatin platform is an agile hub of activities switching genes "on" and "off" by regulating positioning of nucleosomes (*blue circles*). The unwinding of the chromatin leaves the transcription start site nucleosome free for transcriptional activities. Modifications of nucleosome histone tails (*blue lines* extending from *circle*) regulate the process, including DNA methylation (*red circles*), serine phosphorylation (P; *yellow circle*), lysine acetylation (Ac; *brown circle*) and lysine methylation (Me; *orange circle*), and nucleosome remodeler complexes protein required for moving nucleosomes (*black oval*)

epigenetic factors. These proteins are known as the writers, erasers, readers, and remodeler proteins and are discussed further below (Figs. 1 and 2) [4]. In general, these proteins are vital to cell maturation as their function in manipulating the epigenome can have profound effects on the proteome and phenotype of the cell. Through functions to add, remove, and interpret the "histone code," the proteins in these four classes are at the core of epigenetic determinants of cellular fate (such as maturation) [3].

Beyond maturation however, alteration of the epigenome by these proteins can also have profound effects during the dedifferentiation that leads to carcinogenesis. Two potential examples of this would include epigenome-based inactivation of tumor suppressor genes or activation of oncogenes [4]. The great promise in targeting therapy toward these epigenetic events is based on their potentially reversible nature. As discussed later in this chapter, the reversibility of these epigenetic events mirrors the flexibility seen in cellular differentiation during development [5]. For example, as mammalian cells mature from pluripotent progenitor cells to a differentiated phenotype, epigenetic control of gene expression through mechanisms such as histone modification, DNA methylation, and changes to noncoding RNA is key to appropriate differentiation. These epigenetic mechanisms are flexible, being modified as cells reach their differentiated states before settling into a more permanent epigenome [5]. Just as the epigenome is modified during development, data is mounting to support the role of epigenome modification in the dedifferentiating process that is the hallmark of the cancer phenotype. Further, once a gene is silenced, it remains heritable in somatic cells.

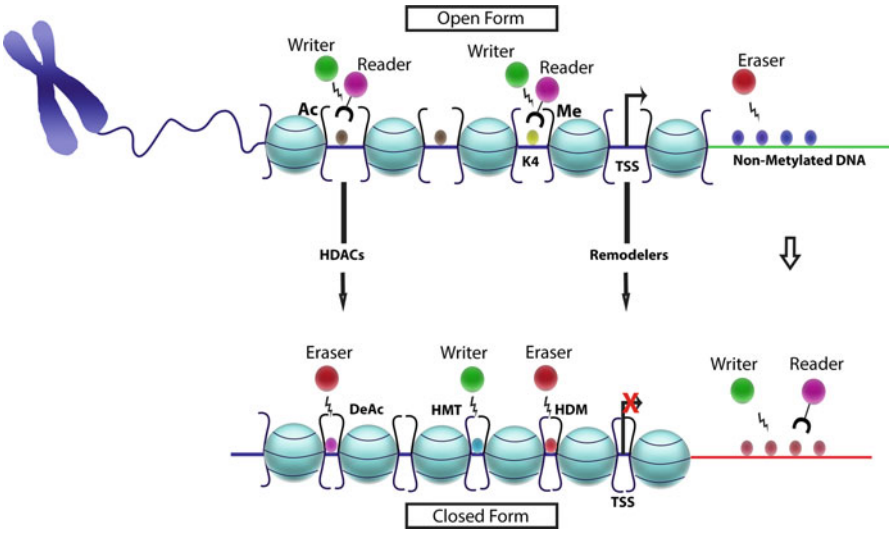

Fig. 2 The epigenomic 4Rs. For open chromatin form (*top*), which exposes the promoter region for transcriptional epigenetic switch in the form of writers (*green circles*), readers (*pink circles*), and erasers (*red circles*), and generally no DNA methylation in associated CpG islands (*yellow circle*). Nucleosomes (*blue circles*) are in an open conformation around the transcription start site (TSS). Writer enzymes in the form of histone methyltransferases (HMTs) add acetyl (Ac), methyl (me), and phosphorylation (P) marks to histone proteins (acetylated lysine, *brown circles*; methylated lysine, *yellow circles*). These regulated chromatin architectural (open and closed form) changes and gene expression regulation. Readers containing specialized domains bind to these distant marks, which are critical for binding to specific modification states. Erasers such as histone deacetylases (HDACs), lysine demethylases (KDMs), and phosphatases are involved in the removal of epigenetic marks. As the chromatin is modulated to the inactive state (bottom), with promoter DNA hypermethylation, it is associated with a more closed form of chromatin near transcription start site (TSS). HDACs, which erase histone acetylation (pink circle), writers (HMTs), which change active histone methylation marks to repressive ones such as H3K9me3 (blue circle) and HDMs, acting as antagonist to HMTs can all impact the epigenome. Another set of writers (DNMT) establish methylation of CpGs at promoter regions (*small red circle*), and readers for this methylation are methylcytosine-binding proteins (MBDs). *Abbreviations*: *HDACs* histone deacetylase, *HMT* histone methyltransferase, *HDMs* histone demethylases

DNA Methylation

DNA methylation refers to the state in which a methyl group (CH3) is bound to a nucleotide on the chromosome. This occurs almost exclusively on cytosine residues that precede guanine in the sequence CpG in the mammalian genome (Fig. 3). Both the distribution of CpG sequences across the genome and the degree to which these sequences are methylated are highly variable [6, 7]. The vast majority of the DNA is relatively poor in CpG density. There are, however, small regions of DNA with highly concentrated repeats of CpG that are known as CpG islands. These islands are frequently found adjacent to gene promoter regulatory sites. The CpG islands adjacent to gene promoter sites remain relatively free of methylation. In stark contrast, CpG dinucleotides in the vast majority of the remaining genome (i.e., not

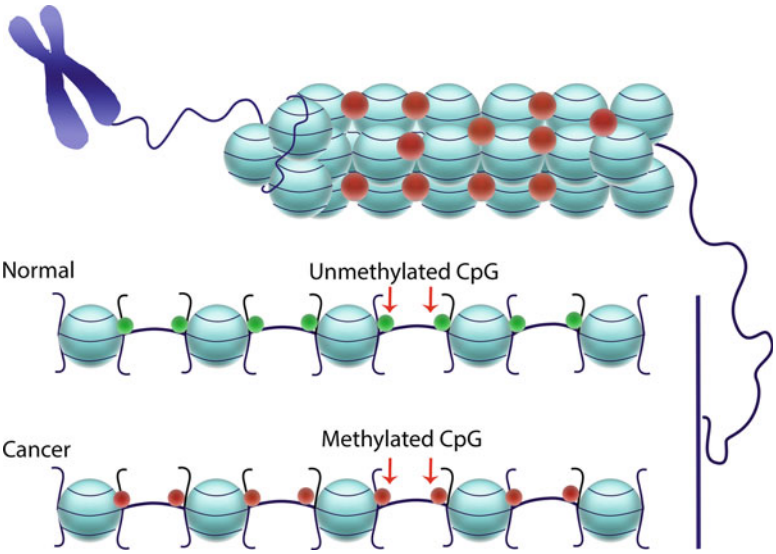

Fig. 3 DNA methylation patterns in normal and disease condition. In a normal cell, the promoter CpG islands (*top*) generally lack CpG site DNA methylation (*green circle*), whereas gene body is heterogeneous for DNA methylation in CpG dinucleotides. In cancer (*bottom*), many genes are heavily methylated in the promoter region of CpG islands, which represses chromatin landscape and leads to abnormal gene silencing. Whereas surrounding region is hypermethylated in the promoter regions with a gain in function

near gene promoter sites) tend to be heavily methylated. This includes heavily methylated areas present at repetitive DNA elements such as Alu (*Arthrobacter luteus* restriction endonuclease-characterized short DNA stretches), long interspersed nuclear elements (i.e., LINEs), and pericentromeric repeats [4, 8].

A growing body of literature is characterizing the effects of CpG island methylation in the cell during embryogenesis, mature cell division, and cellular dedifferentiation found in cancer. The key mechanistic association links increasing methylation of the dinucleotide sequences in CpG islands and decreased gene expression. Methylation-directed gene silencing is critical during embryogenesis, not only directing proper differentiation and maintaining cell lineage but also in ensuring genome stability [5]. Additionally, the phenomenon of gene imprinting, when heritable gene expression is controlled through epigenetic mechanisms (i.e., parental strand-specific expression), is reestablished during this period of embryogenesis [4].

Disorders in methylation can have profound effects on the fate of the cell and host. For example, certain inherited diseases are a result of gene imprinting rather than gene mutation. The neurodevelopmental disorders, Prader-Willi and Angelman syndromes, are two often cited examples of diseases of imprinting [9]. In Prader-Willi, for example, one predominant mechanism is driven by aberrant DNA methylation that silences genes along the maternal allele of 15q11-13 and loss of paternal

genes. This leads to a disorder characterized by mild to moderate cognitive defects (affecting speech, attention, executive function, and mood) that occurs in approximately 1 in 20,000 live births.

As fully differentiated cells divide and renew, opportunities for alterations in DNA methylation profiles exist. As cancer develops, DNA methylation is commonly altered. Fundamental changes in the epigenome include a relative global hypomethylation paired commonly with focal hypermethylation of CpG islands typically in gene promoters [2, 8]. These changes alter the nucleosome structure and global gene expression profiles. Additionally, specific hypermethylation in the promoter region of tumor suppressor genes, such as *Breast Cancer 1 (BRCA1)* or *Von Hippel-Lindau Tumor Suppressor (VHL)*, is commonly encountered and results in silencing of genes critical to the integrity of a cell. It is important to note that once DNA methylation is acquired, it is heritable in somatic cells and can contribute to malignancy [5]. Contemporary research efforts aimed at understanding the hypermethylome of cancer have shown that methylation-associated gene silencing is commonly seen in many tumor types, including colorectal, breast, pancreas, and gastric, amongst others [4, 8]. Generally, hundreds of genes show methylation in many cancer subtypes as demonstrated by the efforts by The Cancer Genome Atlas (TCGA) consortium [10]. Work is now progressing in understanding which of these gene-silencing events are epigenetic drivers rather than simply passenger events.

Beyond the focal hypermethylation, there are associated changes in histone marks including trimethylated histone 3 lysine 27 (H3K27me3), trimethylated histone 3 lysine 9 (H3K9me3), and many others [4]. Finally methylation in selected promoter regions, such as that adjacent to *MutL homolog 1 (MLH1)*, can drive changes to the underlying genome itself. Work by Herman and colleagues demonstrated that *MLH1* promoter hypermethylation drives microsatellite instability in selected carcinomas [11].

Epigenetic Mechanisms in Pancreatic Cancer Carcinogenesis

Original investigations into the role of the tumor suppressor genes, such as *p16*, in PDAC suggested that this family of proteins played a pivotal role in tumorigenesis [12]. Mechanistically, p16 is involved in a cell cycle regulatory complex that functions to arrest the cell at the G1 phase of division. The p16 protein, in particular, is responsible for control of cyclin-dependent kinase 4 (Cdk4) binding to cyclin D1 and subsequent progression through G1. Initial work by Caldas and colleagues found that genetic inactivation was present in 82% of tumors studied [12]. Nevertheless, one-fifth of tumors possessed wild-type (WT) *p16*, which led subsequent investigators to study other potential mechanisms of inactivation of this pathway [12].

The role of gene silencing through epigenetic mechanisms, such as DNA methylation patterns (Fig. 3), was of particular interest in follow-up studies [11, 13]. After confirmation of *p16* WT status in seven PDAC samples, a PCR-based methylation screen targeting the 5′-CpG islands of *p16* was used to investigate the

epigenome. In all but one, homogenous methylation patterns were detected for all *p16* transcripts, which resulted in a loss of downstream p16 protein and subsequent loss of growth suppressor function [14]. DNA methylation patterns were subsequently evaluated in depth for pancreatic cancer. Global methylation profiling assays identified nearly 60 candidate genes, which had altered expression due potentially to changes in methylation [14]. In the same work, candidate methylation markers of gemcitabine responsiveness were also proposed. Subsequent data have similarly shown extensive epigenetic changes in pancreatic cancer with methylation-associated transcriptional activation of many genes that are silenced early during cancer development [15]. These hypermethylated genes are often preferentially poised toward bivalency with both active and silencing histone marks, and environmental pressures may push toward inactivation of many of these genes by DNA methylation [16].

Similarly, the role of the epigenome in oncogene activation has been demonstrated in cell culture and xenograft models of PDAC [17]. Affecting a similar point in the cell cycle, G1-phase progression (as well as G1-S transition), the oncogene *c-myc* is a transcription factor responsible for upregulation of a variety of gene products with function in cell cycle progression, apoptosis, and cellular transformation [18]. In a study by Koenig et al., the regulation of *c-myc* gene expression demonstrated epigenetic changes driven by intracellular calcium concentration that controls the response of the calcineurin/cellular nuclear factor of activated T-cell (NFAT) pathway [17]. Specifically, NFAT binds to an element of the DNA adjacent to a *c-myc* proximal promoter and induces chromatin structural modification to allow for protein-promoter interactions driving c-myc protein translation. Importantly, and in a manner that provides insight into the pharmacologic rationale of targeting the epigenome, the depletion of NFAT abrogated c-myc protein expression leading to G1 arrest and decreased tumor growth in both in vitro and xenograft models of PDAC [17].

While a full review of the epigenetic mechanisms of disease is outside the scope of this chapter, and can be found in detail in chapter ▶ "Epigenetics and Its Applications to the Progression Model of Pancreatic Cancer", it is worth noting that the pancreas methylome clearly plays a role in PDAC [15]. In both in vitro models and patient tumor specimens, Yi et al. showed that cancer-specific promoter DNA methylation for two particular genes, *Basonuclin 1 (BNC1)* and *A Disintegrin-Like and Metalloprotease with Thrombospondin Type 1 (ADAMTS1)*, corresponds with early-stage PDAC [15]. The presence of PDAC-specific methylome changes may in fact hold promise in new early detection (disease-specific biomarker) and treatment paradigms. As such, it is this work in particular that makes a chapter such as this, focusing on epigenetic pharmacology, particularly relevant [15]. Lastly, there are important germ line mutations of critical regulatory elements of the epigenome that occur with some frequency in pancreatic cancer [19]. For example, the *AT-Rich Interaction Domain 1A* (*ARID1A*) gene is frequently mutated in many cancers of gastrointestinal cell origin, including from 2% to 8% of pancreatic tumors, and suggests that aberrant chromatin remodeling in this disease may be driven in part by acquisition of somatic mutations [19].

Pharmacological Strategies

Though there is clearly interplay and cross talk between the various effectors of epigenetics, for the purposes of a pharmacologic discussion, these will be addressed independently. It is important to remember, however, that the mechanisms of action for many of the agents discussed in the ensuing section are multifaceted. To facilitate discussion and understanding, a list of commonly researched agents and their current research point/approval status is noted in Table 1.

Table 1 Commonly researched agents, the current status of research and approval status if applicable

	Drug	Preclinical / Early phase	Approved / Disease
Single Agents			
DNMTi	Azacitidine		Approved/MDS
	DAC		Approved/MDS
	SGI110	Phase I/II	*MDS, AML, Ovarian, hepatocellular, Colon*
HDACi	Vorinostat		Approved/CTCL
	Romidepsin		Approved/CTCL
	Valproic acid		Approved/CTCL
	Pivanex (AN-9)	Phase I/II	*CLL, NSCLC*
	Entinostat	Phase I/II	*Approved/AML, MDS*
	Panobinostat	Phase III	*Hodgkin's Lymphoma, Kidney Cancer*
	Belinostat	Phase I/II	*Relapsed or refactory acute myeloid leukemia*
	Givinostat	Phase I/II	*Chronic myeloproliferative neoplasms*
	Pracinostat	Phase I/II	*AML, MDS, Metastatic sarcoma*
	Panobinostat	Phase III	*Hodgkins Lymphoma, multiple myeloma*
	Rocilinostat	Phase III	*Multiple Myeloma, CRC, Melanoma*
HATi	Curcumin	Phase III	*Breast Cancer, CRC, multiple myeloma*
HMTi	Tazemetostat	Phase I	*ALL, MLL*
	EPZ-5676	Phase I	*NHL, Breast cancer*
	GSK126	Preclinical	*Hematological Malignancies, NHL*
BETi	GSK525762	Phase I	*NUT midline carcinoma*
	JQ1	Preclinical	*AML, Multiple myeloma, NUT midline carcinom*
Combination			
Epi-Chemo	Vorinostat/5FU/Leucovorin		*CRC*
	SGI-110/Irinotecan		*CRC*
Epi-Immune	Aza/Romidepsin/PD-1		*CRC*
	SGI-110/GVAX/CY		*MDS*
Epigenetic priming with other drugs	AZA/Entinostat		*NSCLC, CRC*
	Romidepsin/Aza		*NSCLC*
	Radiotherapy/Vorinostat		*GI cancer*
	Vorinostat/Gemcitabine/paclitaxel/Sorafenib		*Pancreatic Cancer*
	Valprolic acid/hydralazine/Cisplatin		*Cervical Cancer*
	Vorinostat/Capecitabine/Cisplatin		*Gastric Cancer*

Targeting the Effectors of DNA Methylation

In general, there are several unique effectors of DNA methylation that play prominent roles in different biologic systems or at different times during cell maturation. While small noncoding RNA can play a role in directing DNA methylation (and is discussed later in this chapter), the family of catalysts that does the majority of work is known as DNA methyltransferases (DNMTs) [20]. These enzymes facilitate transfer of a methyl group from a donor (commonly *S*-adenosyl-L-methionine or SAM) to the 5′ position of the cytosine in CpG elements. Of note for the discussion to follow regarding pharmacotherapy, SAM exists in a balance with *S*-adenosyl-L-homocysteine (SAH). There are three primary DNMTs identified in mammalian studies: DNMT1, DNMT3A, and DNMT3B. Isoforms of DNMT3A and DNMT3B contribute to DNA imprinting and de novo methylation, while DNMT1 appears to be most important in maintenance of methylation [21].

The conserved elements of DNMT across family members appear to include a conserved sequence motif that binds to SAM [21]. Similarly, all family members have motifs toward the N-terminus, which serves to localize the protein to its nuclear target. For DNMT1, function includes interaction with the DNA replication complex at the replication fork whereby methylation maintenance is carried out as DNA is newly synthesized [22]. As each methylated CpG dipeptide is replicated, DNMT1 rests at the methylation site, flips the cytosine into its catalytic pocket, and facilitates methyl group transfer from SAM before moving along with the DNA replication complex [22].

Preclinical rationale for manipulation of DNMT family members in oncologic therapy is derived from several early studies to elucidate function of the protein. Following discovery of the gene, studies investigating function in cell lines demonstrated that mutation of DNMT1 caused no noticeable changes in embryonic stem cells [23]. Drastically, however, when a similar mutation was bred into the germ line of mice, a uniformly lethal phenotype was obtained. This initial work demonstrated that DNA methylation via DNMT1 function was both necessary and sufficient for preserved in vivo cellular maturation.

Interestingly, further work on methylation has demonstrated the agility of these enzymatic complexes. For example, when studying methylation after replication of X chromosome in cells passaged in tissue culture models, Riggs et al. demonstrated that omissions and errors occurred in as many as 5% of sites for each cell division [24]. These data raised the rational interest in targeting methylation as an oncologic therapy for several reasons. First, the tumorigenesis model whereby spontaneous epigenetic changes may impact phenotype alongside genetic mutations was recognized. Second, the flexibility of cellular processes controlling methylation and subsequent gene expression was proposed to be more "accessible" (or targetable) than corresponding changes in the underlying genome.

Given that initial studies associated oncogenesis with tumor suppressor gene hypermethylation, initial attempts to target DNMT function have focused on inhibition of the protein. Compounds found to inhibit DNMT can be broadly divided into

two categories: nucleoside analogs and non-nucleoside inhibitors [25]. The first generation to be discovered was nucleoside analog compounds initially believed to function as antimetabolites in cytotoxic regimens for leukemia [26]. The hypomethylation that results from therapy with two analogs of cytidine, 5-azacitidine and 2′-deoxy-5-azacitidine (DAC), was discovered after cellular differentiation was noted as a by-product of treatment in embryonic cell line studies [26]. Work to clarify the mechanism of action of these two agents has subsequently been elucidated. After entry into the cell, azacitidine and DAC are incorporated into the RNA and DNA of proliferating cells and recognized by DNMT during replication. Rather than catalyzing methylation, DNMT is irreversibly bound to the nucleotide analog due to substitution of nitrogen for the standard carbon on position 5 of the ring [25]. The differences between azacitidine and DAC are due to their molecular makeup. Azacitidine is a ribonucleoside that is incorporated preferentially into RNA rather than DNA. DAC, in contrast, is a deoxyribonucleoside and can only incorporate into DNA. These compounds both tend to have different mechanisms with different doses. Traditional use with high-dose administration causes direct cytotoxicity due to antimetabolite and DNA intercalation effects. In contrast, low-dose administration has been shown to effect demethylation with little cytotoxicity [27].

The US Food and Drug Administration has approved both azacitidine and DAC for the treatment of myelodysplastic syndrome and certain classes of lymphoma. Additionally, in the European Union, DAC is approved for acute myelogenous leukemia. Work by Silverman and colleagues in hematologic malignancies has shown us that the efficacy of these drugs is slow and responses are seen after several months [28]. As such, testing the efficacy of these epigenetic drugs in solid tumors has to be done carefully with the caveat that current clinical trials are performed in advanced cancers in patients who are rapidly progressing.

Utility of these compounds in solid tumors is under active investigation, but results have been hampered by early use of high doses of these drugs in the paradigm of using maximally tolerated doses similar to cytotoxic drugs and the resultant frequent side effects on bone marrow suppression from high doses [27]. However, in recent years low doses of these compounds have been tested in some solid cancers. Recently the Stand Up To Cancer/AACR consortium funded several trials with combination epigenetic therapy with a DNMT inhibitor, 5-azacitidine, along with an HDAC inhibitor entinostat in lung, colorectal, and breast cancers (discussed in detail below). In pancreatic cancer, for example, there is a wealth of preclinical data that suggests promise for DNMT inhibition either as a single agent or in multi-agent combination therapies. In cell culture models, administration of DNMT inhibitors has been repeatedly demonstrated to have profound effects on cellular growth and tumorigenicity of pancreatic cancer stem cells [29–31]. Additionally, preclinical models suggest a profound sensitization to other cytotoxic chemotherapeutics can be conveyed by low-dose DNMT inhibition. Telomerase activity, critical for cellular immortalization, has also been shown to be impacted by DNMT inhibition [32]. Finally, in vivo testing of DNMT inhibition has validated much of the data from cell culture

experiments: slowing progression of PDAC, extending survival, and sensitizing tumors to combination therapy [33].

A recent search of clinicaltrials.gov notes two trials evaluating the efficacy of DNMT inhibition in human subjects with pancreatic cancer. The first, NCT01845805, evaluates azacitidine in a phase II setting as monotherapy (versus an observation control) after completion of adjuvant therapy in resected pancreatic adenocarcinoma. First opening in April 2013 through the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, this trial is due to accrue 80 patients. The intended patient population for this trial includes those with node-positive disease, margin-positive disease, and/or elevation in CA 19-9. The second trial, NCT02847000, evaluates DAC in combination with tetrahydrouridine (to improve biodelivery) in a phase 0/I setting. Accrual for this second trial has not yet begun. Studies in pancreatic cancer so far have been limited with single-agent therapy given the rapidly aggressive nature of the disease and the slow onset of action seen with these compounds.

The toxicities that are encountered when using cytidine analogs are well documented from use in other settings. In general, there are two distinct profiles that arise from azacitidine and DAC therapy and depend on dose. At high dose, myelosuppressive effects are most common and reflect the cytotoxic antimetabolite profile that characterized their early discovery and use [27]. Importantly, however, the goal of epigenetically directed therapy is to avoid overt cytotoxicity by using low-dose therapy [27]. In these settings, the frequency of side effects are few and morbidity is low [34]. Ongoing work with second-generation nucleoside analogs (such as the DAC prodrug, guadecitabine or SGI-110) aims to increase bioavailability, limit cytotoxicity at higher doses, and improve efficacy [25, 35]. An initial trial testing guadecitabine in hematologic malignancies has shown promising bioavailability of this drug [35].

Non-nucleoside analogs are also of interest in epigenetic drug discovery. While sharing the core mechanism of action, inhibition of DNMT, non-nucleoside analogs do not require DNA intercalation to exert pharmacologic effect. In general, the majority of compounds in this class were discovered to have effects on the methylation profile of cells as a secondary finding [25]. Examples of compounds include certain flavonoids, hydralazine, procainamide, and curcumin. Each compound, or compound family, is purported to have their own distinct mechanism of action. For flavonoids, an indirect effect due to catechol-*O*-methyltransferase (COMT)-mediated accumulation of *S*-adenosyl-L-homocysteine (SAH) is thought to cause DNMT inhibition from SAM/SAH disequilibrium [25]. Hydralazine is thought to be a direct enzyme inhibitor through binding of the active site of DNMT, though this remains highly debated in the field [36]. In general, the use of flavonoids, hydralazine, and curcumin has all demonstrated the capacity to impact pancreatic cancer cell growth and induce apoptosis in vitro [37].

The efficacy of non-nucleoside analogs in the clinic is also promising, though data lags behind that of their nucleoside analog counterparts. Perhaps the best data are from trials involving hydralazine administration in combination with other

antitumor agents. Combination with valproate, for example, has demonstrated a limited capacity to resensitize patients to chemotherapeutics (a topic which will be discussed further later in the chapter), and hydralazine monotherapy was associated with reestablishment of tumor suppressor gene expression in otherwise untreated cervical cancer [36]. To date, there are no ongoing clinical trials evaluating the efficacy of non-nucleoside analogs for the prevention or treatment of pancreatic cancer.

Targeting the Effectors of Chromatin Structure and Function

The structure of chromatin can vary based on the markers which are affixed to the individual histone protein. These conformal rearrangements can dramatically alter the function of chromatin, including its capacity to bind nearby structures such as adjacent chromatin or nearby DNA strands. Based on this structure and function, the expression of genes can be regulated. In a simplistic view, the effectors that mark histones and change chromatin function can be divided into four classes. These are sometimes referred to as the "four Rs of epigenetics" and include the *r*emodelers, w*r*iters, e*r*asers, and *r*eaders (Figs. 1 and 2) [4].

These broad categories reflect differences in the function of the various proteins involved. For example, remodelers can be protein or noncoding RNA that often work in complexes to initiate the process of chromatin remodeling [38]. Epigenetic writers and erasers also often function in complexes of larger proteins as the enzymatic catalysts of histone modification [38]. As implied by the name, writers are responsible for labeling the histones with epigenetic marks. This family of catalysts has many members and can mark by facilitating transfer of acetyl, phosphoryl, hydroxyl, methyl, and many other moieties to the histone. In general, the focus of histone modification occurs at the amino-terminal peptide regions that are exposed at the periphery of the chromatin complex. Erasers are a family of enzymatic proteins that remove the marking of histones. Finally, epigenetic readers are responsible for identifying the epigenetic information laid down and facilitating changes in gene expression profiles (Figs. 1 and 2) [4, 8]. Remodelers help to arrange the histone and chromatin structure.

A historical view of epigenetics posited that increased marking of histones resulted in chromatin unfolding and directly correlated with increased gene expression. We now know that the relationship is complex and that both down- and upregulation of gene expression can be seen with histone modification [39]. Nevertheless, research has begun to wade into the nuanced world of these four protein families in attempts to discover new therapies for pancreatic cancer. While all four (remodelers, writers, erasers, and readers) may represent druggable targets, there are certain classes that lend themselves to therapeutic manipulation easier than others. For example, the enzymatic function of writers and erasers has enabled researchers to screen for and identify inhibitors of these enzymes (many of which are clinically approved for use and discussed below) [38, 40]. An additional class of epigenetic pharmacologic agents being studied focuses on disruption of the protein-protein

interactions central to the function of the reader proteins. The bromodomain inhibitors (or bromodomain and extraterminal, BET, inhibitors of reader protein function) are the classic example of this latter class of agents and will also be discussed later in this chapter [41].

Histone Deacetylase (HDAC) Inhibition: The Prototypical Agent for Histone Modification

In the eraser family of proteins, histone deacetylase (HDAC) and histone lysine demethylases are the two major members [40]. While work to target lysine demethylases is limited [42, 43], the HDAC inhibitors are a particularly well-described and well-studied class of medications that act on this epigenetic eraser family of proteins. There are several HDAC inhibitors that are approved for clinical use for various hematologic malignancies including vorinostat and panobinostat (Table 1). The original discovery of this class of agents was made following empiric compound screens for antitumor agents; only subsequently were the mechanisms of action elucidated [44]. Follow-up work has demonstrated that most of these agents have little-to-no sensitivity for targeting individual HDACs (as opposed to the whole class of proteins) and have potent effects on "off-target" enzymes in related classes [45]. Nevertheless, enthusiasm for this pharmacologic class has not waned, and there are currently more HDAC inhibitors in clinical trials than any other class of epigenetic agent.

The effects of HDAC inhibition on tumorigenesis is an area that has grown exponentially over the past decade. Proposed mechanisms of action include a direct effect on cell death via apoptosis and DNA damage accumulation, cell cycle arrest, reversal of dedifferentiation, and enhanced tumor immunogenicity [40]. Induction of apoptosis can occur via both the intrinsic and extrinsic pathway through gene modification of proteins such as the death receptors (DR4, DR5, FAS) and their ligands [46]. DNA damage repair mechanisms can also be fundamentally altered, and the resulting accumulation of errors can lead to apoptosis or autophagy [47]. The same line of investigation also discovered a toxic accumulation of reactive oxygen species was associated with increased DNA damage and proposed a role of HDAC in native metabolic homeostasis. Work on the mechanistic drivers of cell cycle arrest implicated direct transcriptional changes in genes such as *p21*, *p15*, *p19*, and *p57* [40]. Finally, an immunomodulatory component contributing to HDAC inhibitor efficacy was recently suggested after studies of murine models of carcinogenesis found an intact immune system was necessary for antitumor effect [48].

There are several classes of medications with a proposed mechanism of HDAC inhibition. The two broad categories include pan inhibitors (not HDAC isotype specific and with significant "off-target" effects) and inhibitors that purport to target a specific class of HDAC enzyme. The latter are far less common. Historically, hydroxamates and their derivatives were the most common HDAC inhibitors. These agents are composed of three domains: a cap region with surface recognition motifs, an active zinc-binding group that acts to perform its catalytic function, and a nonspecific linker region. Compounds belonging to this class include vorinostat and panobinostat. These agents generally target several classes of HDAC in addition

to having effects on other cellular lysine deacetylases that act on both nuclear and cytoplasmic protein targets [40, 45]. The nonspecific nature of these agents is principally due to the relative availability of the catalytic domain when these compounds are in their native forms.

The second class of HDAC inhibitors belong to a family known as the benzamides. These agents are characterized by more complex cap and linker regions which increase specificity of binding and limit the activity of the zinc-binding group for a particular HDAC class (generally class I HDAC). The most commonly studied agents in this family of medications are entinostat and mocetinostat [40, 49]. Novel compounds in this family are being frequently described and tested, such as the HDAC class 3 inhibitors RG2833 and RGFP966 [40]. Finally, other attempts to develop HDAC-specific therapies involve agents that architecturally abandon the traditional cap-linker-zinc catalyst mold of prior generations of agents. Thiol derivatives, which shroud the zinc-binding region within a complex ring structure, are one example of this class. The most well-described agent in the thiol class is romidepsin [40, 50].

The clinical utility of HDAC inhibition is limited thus far to patients with hematologic malignancies. Vorinostat, for example, has demonstrated modest efficacy in the treatment of refractory cutaneous T-cell lymphoma [51]. In this supporting work, 8 of 33 patients achieved a partial response with a median time to disease progression beyond 6 months in heavily pretreated patients. These findings, along with work done by many other groups, warranted granting of approval for use in this disease by the United States Food and Drug Administration [40]. The study of other HDAC inhibitors, such as romidepsin and belinostat, has also led approval of these agents for clinical use in selected hematologic malignancies [52]. A recent comprehensive review of HDAC inhibitor trials notes that over 350 clinical trials are currently ongoing to evaluate the efficacy of these agents, with most focused on hematologic tumors [40].

Belinostat is an interesting case study that represents a novel process of clearance for clinical use: accelerated approval. In July 2014, the FDA granted accelerated approval to belinostat (a relatively nonspecific HDAC inhibitor) for relapsed or refractory peripheral T-cell lymphoma [53]. The dose was chosen through a standard phase I dose escalation study that characterized the common side effects of nausea, vomiting, fatigue, fever, and anemia. As a monotherapy in second line or beyond disease, belinostat was found to convey an overall response in approximately one-quarter of patients. Given the accelerated approval paradigm, the end points of overall or progression-free survival were not reported. Importantly, this agent was never tested against control in any of the pre-approval trials, and as such a comparison end point of overall or progression-free survival would be inappropriate (and was not used to determine FDA status). Finally, subsequent studies of combination therapy of belinostat (and other HDAC inhibitors) with CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) in early-phase clinical trials have been encouraging [54]. This experience clearly reflects the dire outcomes in relapsed and refractory peripheral T-cell lymphoma. The parallels (regarding the devastating

prognosis) with advanced pancreatic adenocarcinoma are glaring, and interest in accelerated approval for novel therapeutics in that disease is growing.

The use of these agents in solid tumors is still investigatory, though early reports are promising in selected diseases and when HDAC inhibition is combined with other agents. In breast cancer, for example, there is mounting evidence that targeted epigenetic therapy with HDAC inhibitors can reestablish sensitivity of tumors to antiestrogen therapy. This work was spearheaded in part by Merino and colleagues after successful results from early-phase clinical trials [55]. There are currently trials actively recruiting subjects in phase III for entinostat in combination with an aromatase inhibitor for patients who develop endocrine therapy resistance [56]. This trial is not alone as a recent search of clinicaltrials.gov reveals over a dozen trials registered testing entinostat in breast cancer, with correlative translational research providing clues to the underlying mechanistic rationale for treatment response or failure. Recent work from another of these trials suggests that combination therapy with immunomodulatory agents may be a rational strategy [57].

Combination therapy may be efficacious in other solid tumor models. Data from a phase I/II trial at Johns Hopkins University found that combination epigenetic therapy with azacitidine and entinostat produced responses in some patients with refractory advanced non-small cell lung cancer [58]. Data showed a median survival of 6.4 months in heavily pretreated patients, more than 2 months longer than historical controls. Of the 45 patients enrolled in the study, all of whom received the epigenetic treatment, 19 were able to undergo subsequent chemotherapy, and several had positive responses to treatment. In all, seven patients remain alive, including two who began the therapy nearly 4 years ago. Two other notable results combining azacitidine and entinostat include a phase II trial in advanced breast cancer (NCT01349959) and a phase II trial in metastatic colorectal cancer (NCT01105377) which have recently been completed as part of the Stand Up To Cancer consortium. The breast cancer trials included randomization by hormone receptor status and an optional continuation arm to investigate if epigenetic therapy can resensitize hormone-resistant patients to therapy [59].

In pancreatic cancer models, there has been little published to date suggesting that HDAC inhibition is a viable single-agent strategy for in vivo tumor response [38, 40]. This is despite growing in vitro data suggesting that HDAC plays an important role in pancreatic cancer cell growth, apoptosis, and downregulation of selected tumor suppressor genes [60]. Recapitulating the models developed in other tumor systems, there is in vitro evidence to suggest that combination strategies with HDAC inhibition and nucleoside analogues are promising in pancreatic cancer [61]. In this work by Arnold et al., vorinostat treatment of three pancreatic cancer cell lines resulted in cell cycle arrest and gemcitabine sensitization that appeared to be *p21* dependent.

There are other compounds that demonstrate histone acetyltransferase inhibition that are also worth noting. Many of these are derivatives from natural compounds such as curcumin, anacardic acid, and garcinol [4]. Other compounds, such as BIX-01294, chaetocin, and 3-deazaneplanocin A (i.e., DZNep), can be included in the

category of histone methyltransferase (HMT) and histone demethylase (HDM) inhibitors and are at various preclinical stages of development [8].

Targeting the Reader Proteins, a Relatively New Approach

The importance of the reader proteins in the structure and function of chromatin was highlighted by the discovery of mutations in the PHD domain (plant homeodomain – Cys4-His-Cys3 motif). PHD fingers are involved in chromatin-mediated gene regulation. Co-effectors of this function include the transcriptional coactivators p300 and CBP, polycomb-like protein (Pcl), trithorax group, the Mi-2 complex, the corepressor TIF1, the JARID1 family of demethylases, and many more [62]. Specific mutations in the PHD finger have been found to abrogate the protein's ability to bind protein effector partners and result in various disease conditions including carcinogenesis and immunodeficiency syndromes [62]. Thus, chromatin readers give us a unique opportunity for targeted therapies.

The best example of targets in the reader family of proteins are the bromodomains and extraterminal (BET) family of proteins. In brief, BET protein studies demonstrate a range of activity with the capacity to impact molecular function across a wide array of cellular processes [63]. They not only interact with the chromatin but also seem to function alongside other core nuclear protein complexes to affect DNA damage repair and transcriptional regulation. These findings have paved the way for the identification of potential BET bromodomain inhibitors as novel anticancer agents. Currently three BET inhibitors (I-BET762, JQ1, and I-BET151) are currently in preclinical models [4, 64]. These agents have been shown to bind to BRD2, BRD3, and BRD4 with a capacity to inhibit their engagement with acetyl-lysine residues. To date, effective antitumor properties have been demonstrated in several murine models of carcinogenesis and nearly two-dozen clinical trials are underway in a variety of advanced malignancies as tracked by clinicaltrials.gov.

Targeting the Associated Complexes in Epigenetics: Noncoding RNA and Protein-Protein Interaction

The role of ancillary pathways of epigenetic control to complement DNA methylation and histone modification is a relatively recent discovery. For example, it is becoming more evident that noncoding RNA plays an important role in the regulation of epigenetic processes [65]. In contrast to the central dogma of molecular biology, wherein RNA is supposed to code for amino acids, this family of nucleotides contains members that impart direct effects on cellular function or phenotype without translation into protein. These RNA transcripts are variable in length and can function both within the nucleus and in the cytoplasm. Effector functions of noncoding RNA can vary from epigenetic control (including chromatin remodeling or direction of methylation) to direct gene expression through transcriptional control and binding of DNA or posttranscriptional processing [66]. Examples include tRNAs, snRNAs, miRNAs, siRNAs, piRNAs, tiRNAs, spliRNAs, and sdRNAs among others. In general, the letters preceding RNA in each family provide clues

as to function. For example, siRNA tends to have a gene-silencing function. There are several key transcripts with known function via epigenetic mechanisms of control: Kcnq1ot1, Airn, Xist, and HOTAIR, for example [66]. Importantly, however, the role of microRNAs can be broad as nonspecific binding and "off-target" effects are as likely with this mechanism (as they are with other mechanisms of epigenetic control).

Perhaps one of the first studies to establish a potential role for noncoding RNA in oncogenesis was performed by Yu et al. and published in 2008 [67]. In this work a leukemia model of tumorigenesis was used to demonstrate the power of antisense RNA to silence tumor suppressor gene function. Specifically, with exogenous overexpression of an antisense noncoding RNA targeting *p15*, investigators demonstrated decreased gene expression and increased tumor growth associated with heterochromatin formation and DNA methylation [67]. A translational link was provided in that natural expression of this antisense construct appeared to be associated with decreased *p15* expression from patient samples.

There is strong preclinical rationale to support the role of noncoding RNA transcripts in solid tumors such as pancreatic cancer. First, global transcriptome analyses suggest that as many as 70% of all genes are susceptible to silencing through the effects of naturally occurring siRNA products present in nearby genetic code [68]. Second, members of another noncoding RNA family have already been shown to have effects on the development of pancreatic cancer [69]. MicroRNAs (miRNAs) are generally short RNA transcripts with the capacity to alter gene expression through any of the mechanisms described above. In pancreatic cancer, miRNA-17-92 has been suggested to be a key molecule in the restriction of tumorigenesis of cancer stem cells [31]. Interestingly, the discovery of this link was made after analysis of cancer stem cells' response to therapy aimed at targeting another epigenetic mechanism of gene expression, methylation through DNMT1. Another suggestion of the role that microRNA plays in pancreatic cancer derives from classic high-throughput discovery, necessity, and sufficiency experiments performed in cell line studies of pancreatic cancer [69]. These authors used a methylated DNA immunoprecipitation chip assay to discover that miRNA-615-5p was hypermethylated and silenced. Overexpression of this particular microRNA led to growth inhibition and decreased migration and invasion. Mechanistic studies suggested that miRNA-615-5p acts through effects on insulin-like growth factor 2 (IGF2), itself a heavily imprinted gene that is subject to epigenetic control. The direct influence, whether epigenetic, transcriptional, or posttranscriptional, between miRNA-615-5p and IGF2 is not clear, though the driver of expression (or silencing) of the actual microRNA is clearly through epigenetic mechanisms.

Drug Resistance in Pancreatic Cancer: An Epigenetic Problem?

There are four core mechanisms that have been proposed for acquired drug resistance in cancer therapy: reactivation of an oncogenic pathway, activation of parallel signaling pathways (i.e., bypass mechanisms), pathway-independent tumor cell

growth, and secondary alterations in the targets of selected drug therapy [70]. Classically, these have been described as mechanisms driven by genetic drift in tumorigenesis. It is increasingly being recognized, however, that epigenetic mechanisms of acquired resistance to therapy are important [71]. It is plausible that the relatively quick changes in cancer phenotype that occur during development of therapeutic resistance are driven more by the quick and directed epigenetic mechanisms of gene expression rather than the relatively slow and undirected process of acquired novel gene mutations [71]. Preventing or reversing these epigenetic mechanisms of acquired resistance could lead to more effective systemic therapy and extend survival [6, 71].

In pancreatic cancer there are two core bodies of work that support the hypothesis of epigenome-controlled therapeutic resistance. The first, led by Qin and colleagues, investigated the patterns of resistance that develop in pancreatic cancer cell line models to treatment with gemcitabine (until recently, the gold standard monotherapy in pancreatic cancer) [72]. Results demonstrated a cellular phenotype with dramatically upregulated expression of the 14-3-3σ protein. This protein is one member of a family that is known to bind a number of signaling proteins including key oncogenic effectors. Crucially, the σ isoform has been associated with particularly poor prognosis in pancreatic adenocarcinoma [73]. Mechanistic work to uncover the driver of 14-3-3σ overexpression implicated epigenetic regulation as the root cause. Under gemcitabine therapy, *14-3-3σ* is demethylated by DNA methyltransferase 1 and ubiquitin like with PHD and ring finger domains 1 (Uhrf1) [72]. When gemcitabine therapy was suspended, the epigenome partially reverted to its previous state of heavy methylation of *14-3-3σ*. These findings implicate epigenetic control of gene expression in the acquisition of therapeutic resistance and highlight the promise of targeted epigenetic therapy in combination treatments for this disease.

The use of combination chemotherapeutics using epigenetic agents with standard chemotherapeutics is beginning to show promise in selected tumor systems. As mentioned previously for breast cancer, the combined use of entinostat with all-trans-retinoic acid (ATRA) and doxorubicin resulted in significant tumor regression in xenograft modeling [55, 59]. This work has consequently led to clinical trials that are ongoing, including one successful phase II and an ongoing phase III trial [59]. Additionally, in ovarian cancer patients with platinum-resistant tumors, administration of low-dose 5-aza-2′-deoxycytidine was associated with resensitization to platinum agents (improved objective response rates and progression-free survival) which has led to an ongoing phase III trial (NCT00477386) [74]. Finally, work at Johns Hopkins in heavily pretreated metastatic colon cancer is now trialing guadecitabine (SGI-110) with irinotecan versus standard of care in a randomized phase II setting (NCT01896856). These trials reinforce the notion that future work in PDAC will focus on combination therapy utilizing epigenetic pharmacotherapy with standard cytotoxic, immunotherapy, or future targeted approaches [65].

Future Directions

While current epigenetic therapeutic approaches in solid tumors have showed minimal responses, the future for this therapy remains full of potential. Previous research, focused mainly on the effect of changes in DNA sequence on drug efficacy, failed to account for the changes in the proteome that were not driven by mutational burden. An increasing recognition of the importance that epigenetic factors play on disease biology and treatment response is driving current research. There are several barriers that remain, however, including a deeper understanding of the biology of the epigenome, a recognition of which epigenetic players are targetable and which are bystanders, and the pharmacodevelopment of novel compounds.

Additionally, the integration of targeted epigenetic therapies into clinical patient care will require multidisciplinary cooperation. Similar to data supporting multimodality treatment (surgery, cytotoxic chemotherapeutics, and radiation therapy) to maximize outcomes in pancreatic cancer, the goal of future epigenetic therapeutics will be to integrate novel drugs into a clinically relevant treatment model to allow for continued multidisciplinary care. In this respect, one would expect that epigenetic therapy should be well tolerated with few side effects. This is in keeping with work described earlier in this chapter in which maximal epigenetic benefits could be achieved at relatively low, noncytotoxic doses. Other than the aforementioned approaches, hormone therapy, immunotherapy, and other molecularly targeted therapies may change the landscape of treatment for pancreatic cancer in the future, and it is imperative that epigenetic therapies "play nice" with these other novel treatments as well.

Finally, it is well recognized that pancreatic ductal adenocarcinoma is a disease in need of better biomarkers. This would aid in both the early detection of disease and determining an optimal treatment paradigm. The traditional model of characterizing patient disease largely ignores the underlying biology of a patient's tumor and relies instead on needle biopsy for histopathologic diagnosis, blood measurement of a cell-surface carbohydrate (CA19-9), and imaging. One could certainly envision a future where a more robust analysis of disease biology is performed at key points in a patient's course of disease (from diagnosis to key points in treatment algorithms and therapeutic switches). It is becoming increasingly evident that an analysis of the epigenome would provide valuable data in this future paradigm.

Conclusion

Epigenetic influence on oncogenesis is becoming accepted as an increasingly important aspect of disease onset and progression. The biology responsible for epigenetic control is now becoming clear with key underlying mechanisms that include DNA methylation, histone modification, and noncoding RNA interactions. With clarification of the mechanisms, proteins involved are being characterized with increasing

detail. Targeting of key players is already in use in the clinic for certain tumors, and work is ongoing to broaden the utility of these FDA-approved agents. Importantly, epigenetic targeting appears to have a key role in both direct cellular cytotoxicity and in maintaining tumor response to current chemotherapeutics. As such, the future role of targeted epigenetic therapy in pancreatic cancer will likely include a multimodality approach and take advantage of improving surgical, cytotoxic chemotherapeutic, and radiotherapeutic advancements.

Cross-References

- ▶ Emerging Therapeutic Targets in Pancreatic Adenocarcinoma
- ▶ Epigenetics and Its Applications to the Progression Model of Pancreatic Cancer
- ▶ The Molecular Pathology of Precursor Lesions of Pancreatic Cancer

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Precision Medicine Based on Next-Generation Sequencing and Master Controllers

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Abstract

Despite decades of research and efforts at improving survival, pancreatic ductal adenocarcinoma (PDA) has become the third leading cause of cancer-related deaths in the United States. In fact, by 2020, it is projected to become the second leading cause of cancer-related deaths in the United States. Personalized, or precision, medicine has resulted in improving patient outcomes in other tumor systems. However, for pancreatic cancer patients, there are a limited number of evidence-based targeted therapeutic options that are currently available. Significant advances in DNA sequencing technology have resulted in the identification of a number of genetic mutations and the delineation of core signaling pathways important in PDA. This has subsequently resulted in an advanced understanding of the genetic drivers of the progression of this disease. Facile sequencing technology has moved the field closer to a personalized approach to treating pancreatic cancer. Improvements to the personalized therapy approach will likely result from several factors including the delivery of tumor sequencing results in a clinically relevant timeframe, the development of better targeted drugs, and perhaps a molecular-targeted approach to aspects of PDA biology beyond mutations in the deoxyribonucleic acid (DNA). These advances will allow clinicians to enroll patients in appropriate-matched clinical trials in a timely manner. In this chapter, the opportunities and limitations of a targeted, personalized approach to treating PDA will be discussed.

Keywords

Precision medicine · Targeted therapy · Pancreatic cancer

Introduction

Pancreatic ductal adenocarcinoma (PDA) remains a largely deadly disease with a 5-year survival of only 9% for all stages combined [1]. Currently, it is the third leading cause of cancer-related deaths in the United States and it is on pace to become the second leading cause by 2020 [2]. This mortality rate is due to a number of factors such as aggressive tumor biology; lack of early screening and prevention strategies; and ineffective targeted treatments. Thanks to large-scale high-throughput sequencing studies, our understanding of the molecular driving events in pancreatic tumorigenesis has increased over the past few decades. However, unlike in other cancers, this has not resulted in a similar increase in effective targeted treatment options that are available in the clinic. In fact, the mainstay of pancreatic cancer treatment remains largely conventional and includes surgery for the minority of patients who are diagnosed with resectable disease, and cytotoxic therapy [3–5].

The clinical aggressiveness observed in PDA is due, in part, to its cellular complexity and its ability to survive in a harsh tumor microenvironment. These

factors likely contribute to resistance to many therapies. First, PDA is associated with a dense stromal reaction. The tumor mass is composed mainly of the tumor microenvironment, and it includes mostly nonneoplastic cells, such as fibroblasts and lymphocytes, and noncellular connective tissue [6, 7]. Additionally, the PDA tumor microenvironment also includes a vasculature, but this cancer is classically hypovascular [8]. This is evident when these tumors are visualized with contrast-enhanced computed tomography imaging, which shows hypoattenuated lesions when compared to the well-enhancing normal surrounding pancreatic parenchyma [9]. PDAs are also genetically complex. Though common driver-mutations are present in essentially all PDAs (i.e., high-frequency mutations, such as *KRAS*), there are a significant number of low-frequency mutations of which the clinical significance has yet to be determined [10–12]. It is this degree of genetic diversity that increases the complexity when considering targeted therapy. For instance, it is unclear which low-frequency mutations contribute to the tumorigenesis in PDA by allowing the tumor to overcome a selective pressure and whether they confer a growth advantage. Some of these low-frequency aberrations may simply be passenger mutations [13]. Additionally, assuming that low-frequency mutations are important in PDA tumorigenesis, it raises the question whether using targeted therapies that will impact a small subset of patients will result in meaningful improvement in overall outcomes in PDA.

This chapter will provide a basic overview of DNA sequencing technology that is available today and how it has contributed to our understanding of dysregulated pathways in PDA. Current targeted therapies and outcomes of precision medicine-based clinical trials in PDA will be reviewed, along with other potential therapeutic strategies that go beyond the targeted approach.

Advances in DNA Sequencing and Its Implications in PDA

Sequencing in PDA

Emerging technologies in sequencing, such as next-generation sequencing (NGS) or whole-exome sequencing (WES) or whole-genome sequencing (WGS) strategies, have been used in PDA to determine its genomic landscape as well as its pathologic progression from precursor lesions into PDA [14, 15]. NGS is a powerful tool that allows for parallel sequencing of multiple genes in one test. Compared to WES, WGS and determination of copy-number alterations (CNAs) provide a more granular view of the genomic landscape of the tumor. WGS and CNAs allow the measurement of alterations in DNA structure (i.e., deletions, amplifications, insertions, and translocations) and result in an improved understanding of the patterns of chromosomal instability that are often observed in PDA [16, 17].

When compared to other tumors, sequencing of PDA is not a simple exercise. In part, this is due to the characteristic desmoplastic stroma that makes analysis of pure tumor epithelial cells difficult. Some of the ways to circumvent this limitation in sequencing PDAs include developing patient-derived cell lines or using laser

microdissection, both are methods that enrich the tumor epithelial content [18]. Despite these apparent limitations, over 1300 PDA genomes or exomes have been sequenced, which has added to our understanding of the molecular drivers in PDA (Table 1). More current studies that utilize NGS have focused on WGS and more detailed genomic analyses, combined with ribonucleic acid (RNA) sequencing for a better characterization of PDAs [19, 20]. Despite the increase in utilization of

Table 1 Summary of sequencing studies in pancreatic ductal adenocarcinoma

Author, year	Method	Patient tumors	Xenografts	Sequencing	Reference
Jones, 2008[a,b]	Exome	None	24	Germline & somatic	[10]
Yachida, 2010[a–d]	Exome	None	7	Somatic only	[22]
Campbell, 2010[a]	Genome	13	None	Germline & somatic	[23]
Biankin, 2012	Exome	99	None	Germline & somatic	[20]
Wang, 2012[a]	Exome	None	15	Germline & somatic	[24]
Jiao, 2013[e,c]	Exome	23	None	Germline & somatic	[25]
Witkiewicz, 2015[c]	Exome	109	None	Somatic only	[26]
Waddell, 2015[a]	Genome	75	25	Germline & somatic	[27]
Dal Molin, 2015[c]	Exome	8	None	Germline & somatic	[28]
Bailey, 2016[a]	Genome	456	None	Germline & somatic	[19]
Roberts, 2016[f,c]	Genome & Exome	638, 39 respectively	None	Germline & somatic	[29]
Murphy, 2016[b,c]	Exome	14	9	Germline & somatic	[30]
Makohon-Moore, 2017[g]	Genome	4	None	Germline & somatic	[31]
Humphris, 2017	Genome & Exome	180, 205 respectively	None	Germline & somatic	[32]
Scarpa, 2017[g,h]	Genome	102	None	Germline & somatic	[33]

[a]Pancreatic neoplasms with acinar differentiation
[b]Familial pancreatic tumors only
[c]Pancreatic neuroendocrine tumors only
[d]Microdissected cases
[e]Patient derived cell lines
[f]Patient derived xenografts
[g]Matched primary metastatic site
[h]Macrodissected cases

such sophisticated high-throughput studies, novel high-frequency mutations, beyond the key players such as *KRAS*, have not been identified. However, identification of novel pathways and also subtyping PDA has emerged as a promising deliverable of this work [19, 21].

The first extensive WES analysis of PDA was first published in 2008 by Jones and colleagues [10]. This study used samples from 24 human cell lines and xenografts and utilized Sanger sequencing to sequence 20,661 genes. Genetic alterations that were identified were variable and included point-mutations, deletions, and amplifications. The authors were able to reproducibly identify well-described mutations in *KRAS*, *CDKN2A (p16), TP53*, and *SMAD4* in PDA genomes. Reproducible alterations in other genes, such as *ARID1A, TGFBR2*, were identified, but these were found in lower frequencies. Ultimately, the researchers were able to identify 69 genes that were altered in the 24 analyzed samples. Thirty-one of these were further subdivided into 12 core-signaling pathways that were found to be altered in 67–100% of the sequenced samples. Several pathways were found to be genetically altered in 100% of the tumor samples, such as apoptosis and KRAS signaling pathways. This clustering of genetic alterations along with molecular signaling pathways in this first high-throughput analysis provided a practical approach to support this research movement.

Following Jones and colleagues, a number of other studies provided sequencing of PDA samples. A recently published study by Bailey and colleagues would follow Jones and colleagues to provide the next large-scale sequencing data in PDA [19]. Using NGS, the authors performed a whole-genome analysis of 456 PDA samples. They identified 32 mutated genes, which were then grouped into 10 pathways that were consistently dysregulated. Moreover, when expression analyses were performed, they were able to group PDAs into four subtypes: squamous, pancreatic progenitor, immunogenic, and aberrantly differentiated endocrine exocrine. Furthermore, these PDA subtypes were correlated with discernable histopathologic characteristics.

A study by Makohon-Moore and colleagues extended these NGS studies. Utilizing very strict inclusion criteria, the authors evaluated primary tumor and metastatic lesion samples by using WGS of patients that were treatment-naïve [31]. The goal was to determine the degree of genetic heterogeneity between primary tumors and metastatic lesions. This is important since it may impact a patient's response to therapy. For example, if there is significant intratumoral heterogeneity between two different samples in the primary tumor or between the primary tumor and a metastatic lesion, it is likely that a patient would develop early resistance to targeted treatment. However, if the primary tumor is genetically similar to a metastatic lesion, it is plausible that both tumors would be sensitive to the initial therapy [34, 35]. A total of 39 samples were evaluated (26 from metastatic lesions, 3 from different regions of the primary tumor, and normal tissue) in four patients. There was a limited variability of driver mutations in untreated patients with metastatic PDA (mPDA) that were present in the primary tumor and the metastatic lesions. This suggests that in patients with metastatic cancer, there may be a clinical benefit afforded by using targeted therapies geared towards driver mutations in the primary tumor.

Moving forward, the goal is to continue the genetic characterization of PDA, to understand how these genetic aberrations relate to the clinical features of the patient's disease, and to identify therapeutic targets. Moreover, there will be a continued trend and interest to further continue to characterize dysregulated pathways and subtypes of PDA.

Current Clinical Use of Next-Generation Sequencing in PDA and Its Implications

There has been an emergence of studies that attempt to link/associate patients' tumor mutations with currently available targeted therapies. The goal of these studies is to take advantage of the opportunities offered by NGS to characterize genetic pathways that drive a specific PDA and to match it to an available targeted therapy. One such trial has been the Individualized Molecular Pancreatic Cancer Therapy (IMPaCT) Trial from Australia [36]. This was a feasibility trial that aimed to demonstrate the ability to successfully acquire patient samples and to provide quality genomic data for three molecular targets: *HER2* amplification, *KRAS*, and mutations in DNA repair pathways (*BRCA1*, *BRCA2*, *PALB2*, and *ATM*). The goal was to evaluate whether it would be feasible to provide sequencing results in a clinically relevant timeline. Inclusion criteria included newly diagnosed PDA patients who either received one cycle of gemcitabine for metastatic disease or patients who were treatment-naïve. Patients were randomized in a 1:1 fashion and offered standard therapy (gemcitabine) versus personalized treatment (gemcitabine plus targeted therapy) depending on the patient's genetic aberrations. At the time that the results of the trial were reported, no patients were successfully treated on the protocol.

The Pancreatic Cancer Action Network (PanCAN) has also launched the Know Your Tumor® Initiative with the goal of providing sequencing data to patients and their oncologists in order to facilitate the use of targeted therapy or clinical trial enrollment. The initial experience resulted in 117 patient sequencing reports, with the identification of an "actionable" finding in approximately 40% of cancers (actionable findings were defined as the availability of a targeted therapy in an identified molecular abnormality in any cancer type or predicted response based on pathway or mechanism-defined for the identified target) [37]. This resulted in 43% of patients being referred to high-priority clinical trials and 53% were recommended in the direction of off-label targeted therapy [38].

A similar multi-institution trial has been designed and implemented in the United States by the authors (MJP and JRB), with the goal of randomizing 60 patients along standard treatment and molecularly targeted therapy (MTT). This trial is also supported by PanCAN and the American Association of Cancer Research. Sequencing of 600 genes and protein expression analyses will be undertaken to further predict the patient's response to either standard therapy or MTT. The results of this trial are forthcoming. PanCAN has also implemented a multi-institution clinical trial called Precision Promise. Its aim is to promote data-sharing by promoting a number

of substudies that investigate different therapies under the same clinical trial umbrella in an effort to expedite the breadth of targeted therapies available to patients with PDA. A similar trial is being implemented in England, called PRECISION-Panc, where multiple subtrials will be carried out under the same umbrella clinical trial. The goal will be to provide molecular profiling of patients' tumors followed by enrolling patients in clinical trials that utilize the targeted approach [12].

In addition to the trials described, there are a number of currently active trials that are based on identifying genetic aberrations for which targeted therapy is available. These include clinical trials, commonly referred to as basket trials, from the National Cancer institute (NCI) including NCI-Molecular Analysis for Therapy Choice (MATCH) Trial, NCI-Molecular Profiling-Based Assignment of Cancer Therapy (MPACT), and Initiative for Molecular Profiling and Advanced Cancer Therapy (IMPACT 2) [39–41]. These studies are not specific for patients with PDA; however, they are likely to recruit patients with PDA based on the trial designs.

The Use of Targeted Therapy in the Treatment of PDA

History of Precision Medicine and its Role in PDA

One of the earliest examples of precision oncology in clinical practice is the use of imatinib in patients with chronic myeloid leukemia (CML) that harbor the Philadelphia chromosome (i.e., the *BCR-ABL* mutation) [42]. The successful clinical use of mutation-targeted therapies has remained elusive in PDA. Even though the number of US Food and Drug Administration (FDA)-approved mutation-targeted therapies has increased over the years and has resulted in an improvement in outcomes in other cancers, similar results have not been realized in PDA.

In PDA, the use of targeted therapy is limited largely due to the fact that many alterations tend to result in loss-of-function in genes that would traditionally be considered tumor suppressors genes (TSGs). In general, TSGs halt cell proliferation, disrupt the cell cycle, and can initiate apoptosis; therefore, the inactivation of TSGs is a critical event for the progression of tumorigenesis. Since rescue of this genotype would require TSGs to regain function, this limits the use of small molecule compounds or drugs that generally are most effective against inhibiting oncogenes (i.e., in the setting of gain-of-function mutations), and not "turning on" an already "turned off" gene. High-frequency TSG mutations are common in PDAs (e.g., *TP53, CDKN2A*) and limit the personalized approach. At the present time, perhaps the most effective personalized therapy for PDA is targeting the BRCA pathway, which contains genes that are known TSGs, resulting in *synthetic lethality* (see next section). Perhaps the resurrection of gene therapy will become a clinical reality. If this happens, one can imagine real precision therapy, wherein specific TSGs can be sequenced in individual tumors and a matched gene therapy option can be utilized (e.g., SMAD4 overexpression for SMAD4 deleted tumors).

Pathways Dysregulated in PDA and Opportunities for Targeted Therapies

Historically, mutations or genomic alterations of *KRAS, CDKN2A (p16), TP53, and SMAD4* have been implicated in the development of pancreatic intraepithelial lesions which ultimately lead to the development of PDA [43, 44]. More generally, multiple pathways are dysregulated in PDA and, in theory, targeted therapies can be used to exploit their specific function. Figure 1 demonstrates commonly altered pathways in PDA and downstream effectors that play a role in tumorigenesis. The remainder of this section will expand on these pathways and provide an overview of therapeutic strategies and options that can be utilized in patients with PDA. Though there have been mixed results with targeting some genetic aberrations, it is the degree and frequency of dysregulation in key cellular processes that make them ideal therapeutic targets in PDA. Therefore, an understanding of the role of individual pathways that are activated or deactivated in PDA will be instrumental to successfully target them in a personalized manner.

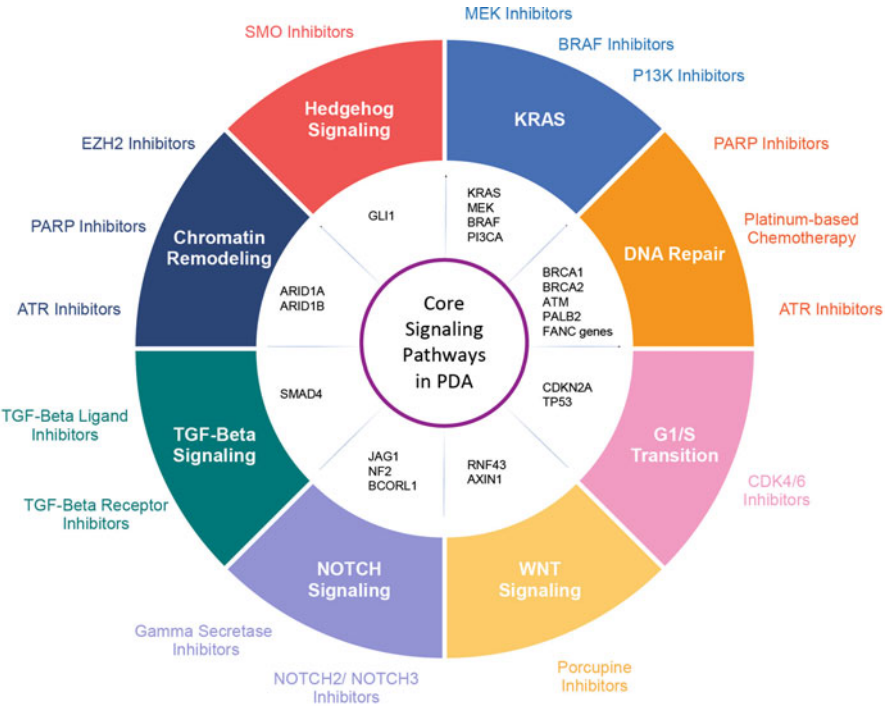

Fig. 1 Core signaling pathways implicated in PDA, genetic aberrations implicated in tumorigenesis, and potential targeting strategies

Targeting KRAS Signaling and Its Downstream Effectors

The *RAS* family of oncogenes consists of *HRAS, NRAS,* and *KRAS* and one or more isoforms of this gene are mutated in most cancers [45]. The *KRAS* pathway is one of the best-characterized pathways in cancer and *KRAS* mutations are frequently observed in PDA, occurring in roughly 95% of tumors [43, 44, 46]. Activity of KRAS is tightly regulated, and under nonpathologic conditions, it exists in an inactive state (i.e., bound to GDP). Extracellular signals, such as growth factors, result in activation of KRAS and the conversion of GDP to GTP and activation of its downstream targets. *KRAS* mutations are found in PDA precursor lesions, so they are believed to occur early in the progression of PDA. Point mutations in *KRAS* often occur in codons 12, 13, or 61 resulting in a constitutively active GTPase that is unable to hydrolyze GTP. This results in sustained signaling of a number of downstream KRAS targets that affect cell survival, proliferation, cell cycle progression, apoptosis, and metabolism [45]. The importance of *KRAS* mutations in the initiation of PDA has been underscored by experiments that utilize genetically engineered mouse models (GEMMs), in which mutant *KRAS* is driven to be specifically expressed in the pancreas [47–50].

Targeting KRAS has been difficult to date, and in fact, KRAS is thought to be an "undruggable" target by some [51]. The NCI has started a program that is specifically geared to the development of KRAS inhibitors [52]. The difficulty with developing a small-molecule to target KRAS is, in part, due to the fact that it has a high affinity for GTP. GTP is abundant in the cell, and it effectively blocks access to the active site of the protein by other small molecules. Targeting farnesylation, one of the post-translational modifications of KRAS which affects its localization to the cell membrane, has not resulted in any significant clinical benefits either [53]. Similarly, specifically targeting the localization of KRAS to the cell membrane, which is dependent on PDEδ, with the use of PDEδ inhibitors has shown some success in xenograft models [54]. Targeting mutant *KRAS* with siRNA has been done in xenograft models, but this has yet to be translated to the clinic [55, 56]. In humans, KRAS siRNA was well tolerated and perhaps even efficacious in patients with locally advanced PDA [57].

Considering the difficulty with targeting KRAS directly, a significant amount of effort has been placed in targeting the downstream effector pathways. KRAS-driven tumors are believed to be dependent on MEK signaling for continued proliferation [58]. Therefore, MEK inhibitors have also been tested in preclinical models with positive results, which have not been reproduced in clinical trials. CI-1040A and AZD6244, two potent MEK inhibitors, have been investigated and were found to be ineffective in patients as second line therapy or as combination therapy with capecitabine in a randomized phase 2 trial [59, 60]. Similarly, trametinib in combination with gemcitabine, when compared to gemcitabine therapy alone, was not found to be superior in a randomized phase 2 trial for patients with treatment-naïve mPDA [61]. These clinical trials underscore the importance of targeting multiple effector pathways simultaneously [62, 63]. For example, concurrent inhibition of MEK and phosphoinositide 3-kinase (PI3K) or AKT may be required to overcome

the limitations of targeting and inhibiting a single pathway [64–66]. This is due to the fact that there are data to suggest that activation of the PI3K pathway results in resistance to MEK inhibitors [64]. However, despite this, a combination of PI3K and MEK inhibition was not associated with increased survival when compared to modified FOLFOX in patients who failed prior gemcitabine therapy [67]. There have also been studies that have demonstrated synergism with the use of EGFR inhibitors and MEK inhibitors, especially in patients with wild-type *KRAS* tumors [68, 69]. Erlotinib, an epidermal growth factor receptor (EGFR) inhibitor, is currently FDA-approved for use as a second line therapy for recurrent, mPDA [70]. Moving forward, the combination of many of these therapies along with new targeted agents may be beneficial. In regards to a personalized approach, targeting *KRAS* mutations would certainly make this an all-inclusive line of therapy (i.e., one size fits all treatment, since the majority of PDAs harbor *KRAS* mutations). However, some investigators are studying whether specific *KRAS* amino acid changes, even at the same codon, might be more targetable than others.

Targeting the G1/S Checkpoint

CDKN2A (p16), a TSG, is another high-frequency mutation in PDA, found in over 95% of tumors [10]. It is a cyclin-dependent kinase inhibitor that functions to stop the transition of the cell from entry into S-phase by inhibiting the kinase activity of CDK4 and CDK6 [71–73]. In PDA, alterations in p16 expression can be due to promoter hypermethylation, homozygous deletions, or single-allele loss with a concomitant mutation in the second allele [74, 75]. All of these lead to inactivation of p16, which subsequently result in increased phosphorylation of Rb-1. This leads to deactivation of Rb-1 and progression through the G1-S cell cycle checkpoint, resulting in increased cell proliferation [74, 76].

In mutant *KRAS*-driven cancers, the loss of p16 is common and results in cell cycle dysregulation. Considering this, there is significant interest in recapitulating the function of p16. However, since *CDKN2A* is a TSG and therapies that result in reinstatement of its expression are limited, there is significant interest in suppressing activity of its targets, CDK4 and CDK6. CDK4/6 inhibitors, such as palbociclib and abemaciclib, have been developed and used in other tumor types and a number of other diseases [77, 78]. In PDA, both in vivo and in vitro studies have shown mixed results with the use of these inhibitors [79–81]. In PDA, inhibition of this pathway is currently being investigated. Actively enrolling trials include a phase I clinical trial evaluating the efficacy of palbociclib and gedatolisib, a PI3K/mTOR inhibitor, in patients with a number of solid tumors, including PDA (NCT03065062) and a phase I dose-escalation study evaluating palbociclib in combination with nab-paclitaxel in mPDA (NCT02501902). Another phase I/II clinical trial is evaluating the safety and efficacy of ribociclib in combination with Everolimus in patients with refractory mPDA (NCT02985125). Lastly, another phase Ib dose escalation trial is evaluating the safety of ribociclib in patients with advanced solid tumors and may recruit patients with PDA (NCT02703571). The results of these studies will be forthcoming.

TP53 is a common TSG mutated in most solid tumors and is mutated in 75% of PDAs [82]. *TP53* is a transcription factor which modulates the expression of genes that are implicated in cell cycle arrest and apoptosis in the setting of DNA damage or cellular stress [83]. Generally, a mutation accompanied with loss of heterozygosity (LOH) in the second allele leads to its inactivation. Once cells lose *TP53* expression, it allows them to bypass the G1-S cell cycle checkpoint, which again, results in increased cell proliferation [76]. Similar to p16, targeting of p53 is difficult since it is also a TSG. Due to this, it has become attractive as a target in tumor immunotherapy. The modified vaccinia virus ankara vaccine expressing p53 (p53MVA) has had some success in preclinical models [84]. Currently, it is being investigated in a clinical trial that includes patients with PDA (NCT02432963), but the success of this therapy is still unknown. A number of preclinical models have attempted to reactivate TP53 with the use of small molecules, such as APR-246 [85, 86]. An ongoing phase II clinical trial is evaluating the efficacy of SGT-53, liposomal nanocomplex tumor-targeting delivery of the wild-type p53 gene, in combination with gemcitabine and nab-paclitaxel in patients with mPDA (NCT02340117). To date, there have not been any clinically relevant therapies that have resulted in recapitulation of TP53 function that have resulted in a clinical benefit for patients with PDA [86, 87]. However, since it is commonly disrupted in PDA, the pursuit of targeting this genetic lesion is a worthy cause [88].

Exploiting BRCAness and DNA Damage Response and Repair Pathways

Genetic alterations in *BRCA1/2* and other DNA damage response and repair genes (the DNA damage repair, DDR, pathway) are observed in 5–17% of PDAs [11, 26]. Furthermore, germline mutations in *BRCA1* and *BRCA2* have been shown to increase a patient's risk of developing PDA 3.5–10-fold [89], as have mutations in the Fanconi anemia genes (i.e., *FANCC, FANCG,* and *FANCN/PALB2*) [90–92]. One of the features of tumors that harbor *BRCA*-related mutations or alterations in the DDR pathways is chromosomal instability [11, 23, 93]. Such mutations have been exploited in ovarian cancer, since tumors that are deficient in DDR have increased susceptibility to platinum-based therapy especially when combined with poly-ADP-ribose polymerase (PARP) inhibitor therapy [94].

This increased susceptibility to platinum-based therapy has been studied in PDA and has shown promising results. Golan and colleagues retrospectively reported on a large cohort of 71 patients with *BRCA1* or *BRCA2* associated tumors. They found that in patients with stage 3 and 4 disease who received platinum-based therapy ($n = 22$), when compared to those who received non-platinum based therapy ($n = 21$), there was improved in median overall survival (22 vs. 9 months, $p < 0.039$) [95].

As a result of the findings from preclinical models and retrospective studies, prospective trials have investigated the utility of PARP inhibitors in patients with PDA and germline mutations in DDR pathways. PARP inhibitors (PARPi) are a class of drugs that cause an accumulation of single-stranded breaks (SSB) in DNA. Once the replication fork encounters a SSB, it may result in termination or the formation of

a double stranded break (DBS); cells that are BRCA-deficient are unable to repair these DSB via homologous recombination leading to cell death through mitotic catastrophe [96, 97]. This is a concept referred to as *synthetic lethality* [98]. A number of clinical trials have either recently been reported or are currently ongoing in order to investigate the safety and efficacy of PARPi in patients with *BRCA1/2* or *PALB2* mutations and have shown encouraging results [99–101]. At the present time, PARPi are perhaps the most promising avenue that utilizes targeted therapy that may be beneficial to a subset of patients with PDA. Further research will need to show whether tumors that harbor *BRCA1/2* mutations are equally as sensitive to PARPi and platinum-based therapy. Moreover, it is important to remember that other genes are commonly mutated in the DDR pathway in PDA. These include *ATM, ATR, RAD51, RAD51C,* and *RPA1*. Identification of these targets has raised the possibility of use of ATM and ATR inhibitors in PDA and in other tumors [102]. In fact, there are a number of preclinical models or clinical trials ongoing that are evaluating the use of these therapies in combination with PARP inhibitors and platinum-based chemotherapy [103–105]. These studies will address the question of whether mutations in the DDR pathway result in the same cancer phenotype. Lastly, to maximize the benefit afforded with PARPi therapy, both alleles must be inactivated. Therefore, the role of NGS is underscored here where reliable sequencing results must be available to clinicians in order to maximally utilize this targeted therapy.

Chromatin remodeling and mutations in SWItch/sucrose non-fermentable (SWI/SNF) nucleosome complex are common in many tumors [106, 107]. The SWI/SNF nucleosome is a complex that consists of ATP-dependent chromatin remodeling factors that control the transcription of a number of genes by altering the chromatin structure [108, 109]. Loss of *ARID1A*, one of the components of the SWI/SNF complex, is the most common event (albeit, one that occurs at an overall low-frequency) and it behaves as a TSG in PDA [19, 110, 111]. Mutations in other subunits of the SWI/SNF complex have also been observed, and these include *ARID1B, SMRCA4,* and *SMRCA2* [112]. Recent studies have demonstrated that the use of PARP or ATR inhibitors results in increased sensitivity in tumor cells that are deficient in *ARID1A* [113, 114]. This preclinical data can be used to expand the use of PARP and ATR inhibitors in patients with PDA who may harbor mutations in the SWI/SNF complex.

Role of SMAD4/TGF-β Signaling

TGF-β signaling has been implicated in pancreatic cancer; a mutation in at least one of the genes in the pathway is present in almost all PDAs [10, 115, 116]. One of the commonly dysregulated genes in this pathway is *SMAD4,* also known as *DPC4,* a TSG that is located on chromosome 18q. It encodes for a transcription factor that plays a role in the transforming growth factor beta (TGF-β) signaling pathway [117, 118]. In PDA, aberrations in *SMAD4* can occur due to homozygous deletions or LOH, coupled with a point mutation that results in its inactivation. Mutations that result in loss of SMAD4 expression are found in 55% of PDAs. Furthermore, mutations in *SMAD4* occur late in the progression of PDA tumorigenesis and are believed to play a role in the metastatic potential of this tumor [15, 119–121].

Targeting of this pathway would be clinically useful, considering the frequency with which it is lost along with other elements of this signaling pathway. Inhibition of this pathway can occur by inhibiting the ligand-receptor interaction with the use of TGFβ ligand inhibitors or with the use of TGFβ receptor inhibitors [122, 123]. The use of these compounds is currently being evaluated in a number of other solid tumors. In PDA, LY2157299, a small molecule inhibitor of the TGF-β receptor I kinase, was evaluated in a phase II double-blind clinical trial in combination with gemcitabine in patients with unresectable PDA. This trial showed an improvement in overall survival and progression free survival with the doublet, with an acceptable toxicity profile [124].

Despite the importance of targeting the loss of *SMAD4*, there have been no synthetic lethal or other targeted therapies that have been used experimentally or clinically to specifically target this molecule. However, due to the pattern of expression of *SMAD4*, especially in metastatic lesions, it has been proposed to serve as a prognostic marker for poor prognosis [125]. There have been some studies that suggest that in patients with locally advanced PDA that exhibit *SMAD4* expression would be suited for chemoradiation, compared to patients with loss of expression of *SMAD4* who may not benefit from such intensified local therapy [126, 127].

Targeting the Wnt Signaling Pathway

Alterations in the Wnt signaling pathway are common in many gastrointestinal malignancies. Perhaps the best example of this is mutation of the *APC* gene and its role in colorectal tumorigenesis [128]. Mutations in the *APC* gene are relatively uncommon in PDA, especially when compared to other genes within the pathway. These include *RNF43, AXIN1/2,* and *GATA6* [129–131]. A number of studies have shown that Wnt signaling is required for the initiation and progression of PDA [131]. Wnt signaling results in expression of β-catenin/TCF4 transcription factor, which in turn results in expression of RNF43. *RNF43* encodes an E3 ligase which is responsible for ubiquitination and degradation of Frizzled receptors [132]. Therefore, mutations in *RNF43* result in constitutive signaling through the Wnt signaling pathway. The difficulty in targeting genes within the Wnt signaling pathway is reflective of our current limitations in targeting TSGs. However, the use of LGK974, which is an inhibitor of Wnt ligand secretion, has shown promising results [130, 133].

Targeting NOTCH Signaling in PDA Tumorigenesis

The NOTCH signaling pathway is important in a number of malignancies, including PDA [134, 135]. The importance of NOTCH signaling in PDA is further established by GEMMs that demonstrate that, in the setting of oncogenic *KRAS*, its activation is necessary for the initiation and progression of PanINs [136, 137]. Moreover, NOTCH signaling has been shown to promote "stemness," epithelial-mesenchymal transition, and chemoresistance [138–140]. And aberrations in expression in the NOTCH signaling pathway have been associated with poor clinical outcomes in patients [141, 142].

Though *NOTCH* mutations are uncommon, studies have shown that other components of the pathway are amplified and result in overexpression [26]. In in vivo and in vitro experiments, there is a strong body of evidence that supports suppression of the NOTCH signaling pathway as therapeutically relevant strategy in PDA [143–146]. Options of inhibition of NOTCH signaling include inhibitors of gamma-secretase, which is required for transduction of signaling through the pathway. More specifically, interactions with the cell-membrane protein NOTCH by one of its ligands initiate proteolytic cleavage of the protein at both its intra- and extracellular sites. Gamma-secretase is necessary for cleavage of NOTCH in the intracellular space. Once NOTCH has been cleaved, it then translocates to the nucleus and modulates the expression of its target genes [147].

The use of gamma-secretase inhibitors has been explored in clinical trials. A clinical trial to evaluate the safety and efficacy of PF-03084014, a gamma-secretase inhibitor, in PDA has been terminated (NCT02109445). Another trial is currently in place, but not actively recruiting, which will evaluate BMS-906024, another gamma-secretase inhibitor, in solid tumors and may accrue patients with PDA (NCT01292655). Another agent, RO4929097, has been evaluated in patients with previously treated mPDA. Though the study showed that this agent was well tolerated in patients with mPDA, development of this compound has been discontinued by Roche [148].

Another strategy for the targeting of the NOTCH pathway includes the use of monoclonal antibodies. This strategy has shown promising results in xenograft tumors in mice when used in combination with chemotherapy [149]. In clinical trials, however, this therapeutic approach has not been as successful. The use of tarextumab (OMP-59R5), a fully human Notch2/3 monoclonal antibody, has been evaluated in a randomized, placebo-controlled, phase Ib clinical trial in patients with untreated mPDA in combination with gemcitabine and nab-paclitaxel and was shown to be well tolerated, safe, and have some antitumor effects [150]. However, when this combination therapy was studied in a phase 2, nonrandomized, placebo-controlled clinical trial, the results did not reveal any improvement in overall survival in patients with mPDA as a first line therapy [151]. Like many potential targeted therapies, the preclinical data to support targeting of the NOTCH pathway are robust; however, the clinical data thus far have not been as promising. This is highlighted by the importance of this pathway in the tumorigenesis in PDA. Improvements in approaches to target components of the NOTCH signaling pathway may result in promising therapies that can become available in the clinic.

Targeting the Hedgehog Signaling Pathway

In mammals, Hedgehog signaling is important in embryonic development and differentiation gastrointestinal tissue. Beyond the embryonic period, it plays a role in tissue homeostasis and has been implicated in the pathogenesis of a number of diseases [152–154]. In PDA, overexpression of Hedgehog is seen early in the development of PanIN-1 s and in preinvasive or invasive epithelium; however, its expression is absent in normal pancreas tissue [155, 156]. Overexpression of hedgehog in abnormal pancreatic tissue depends on expression of oncogenic KRAS, which suggests that Hedgehog is a downstream effector [157]. Yet the question

remains whether the role of Hedgehog is dependent on intracellular signaling alone within tumor epithelial cells, or whether it is as a consequence of aberrant ligand signaling in the tumor microenvironment.

The role of Hedgehog signaling has been extensively studied in mouse models that have helped delineate its mechanism [158–160]. Based on GEMMs, the role of Hedgehog ligand was determined to be important in PDA tumorigenesis. In a study by Nolan-Steveaux and colleagues, a mouse model was generated in which SMO-deficient pancreatic progenitor cells (which are insensitive to Hedgehog signaling) were shown to develop PDA at a similar rate as wild-type SMO controls [161]. Moreover, both the SMO-deficient and SMO-wild type mice developed equivalent expression of the Hedgehog ligand and inhibition of GLI1 in both of the groups resulted in increased apoptosis and decreased cell growth [161]. This model suggested that stromal Hedgehog ligand-dependent signaling and non-canonical Gli signaling in tumor epithelial cells are important in KRAS-dependent PDA tumorigenesis [161].

This finding has been further expanded to focus on the Hedgehog ligand, which is produced by tumor epithelial cells, which results in SMO-dependent activation and signaling of adjacent stromal cells (i.e., cancer-associated fibroblasts, CAF) along a canonical signaling pathway [162]. This leads to desmoplasia – one of the hallmarks of PDA. CAFs and cancer-associated stem cells have been implicated in their role in PDA. Co-culture of tumor epithelial cells and CAFs that have been isolated from PDA results in increased proliferation, colony formation, invasion, and resistance to gemcitabine both in vitro and in vivo [162–165]. Downstream effectors of the Hedgehog signaling pathway, such as SMO or GLI1, are two potential avenues to provide inhibition of this pathway. The SMO-inhibitor, LDE225, has been evaluated in a phase Ib in patients with locally advanced or mPDA in combination with gemcitabine [166]. GDC-0449, also an SMO inhibitor, has shown success in pre-clinical models [167]. However, when this compound was evaluated in combination with gemcitabine in patients with mPDA, there was no improvement in outcomes when compared to gemcitabine treatment alone [168]. Though there is variability in regards to the success of targeting this pathway, there continues to be much interest in targeting the Hedgehog signaling pathway in PDA. Lastly, targeting of cancer-associated stem cells has also been attempted with the use of monoclonal antibody and is currently being investigated with the use of a "cancer stemness" inhibitor, BBI608 (NCT02231723) [169]. Therapeutic strategies, such as this one, provide a unique way to target vulnerabilities in PDA that go beyond genetic alterations.

History of Ex Vivo Modeling and the Importance of Preclinical Models in a Personalized Approach to PDA

Molecular and pathologic studies have established a model for progression of PDA, with oncogenic *KRAS* having an integral role for the inception of tumorigenesis [50]. As discussed in the prior section, a number of genetic aberrations contribute to the tumorigenesis and progression of PDA [47]. The use of genetically engineered

mouse models (GEMMs) has been instrumental in our understanding of the initiation and progression of PDA [47]. Moreover, GEMMs have increased our understanding of the role of the tumor microenvironment in PDA and of ligands that are important in dysregulated pathways [161, 170]. Additionally, preclinical models, such as human cell lines, xenograft tumor models, and patient-derived tumor xenografts, have been used to understand the biology of PDA and to identify new therapeutic targets for patients. An exhaustive discussion of ex vivo models is beyond the scope of this chapter, but two new techniques, discussed below, have the potential to significantly propel targeted therapy in PDA: conditionally reprogrammed cells and organoids.

Conditionally reprogrammed cells are a relatively new technique for tumor modeling that allows for quicker regeneration of patient-derived tumor cells that can be used for drug-sensitivity testing [171, 172]. Most recently, three-dimensional culture of patient derived tissue in the form of organoids has been heralded as the next generation ex vivo culture model for PDA [173]. Mouse- and patient-derived organoids have been derived by a number of laboratories around the world and have been genetically modified using CRISPR technology or have been used to test drug sensitivities [174–176]. Organoids can be established from surgical specimens and from biopsy specimens. This model allows for the establishment of a pure tumor epithelial population of cells that recapitulates the genomic make-up of the initial tumor specimen [177, 178].

There are a number of preclinical models that are available for translational studies that have attempted to recapitulate the genetic diversity that PDAs exhibit. There are pros and cons that are associated with each model, and at the present time, patient-derived organoids represent perhaps the most promising preclinical model that is available to researchers. There are still many questions that need to be addressed with organoids, including whether the genetic complexity that is seen in the primary tumor is maintained in the organoid. Still, this model can result in an improvement in our understanding of the tumorigenesis and the role or low-frequency mutations in the progression of PDA. This model has already been exploited with intestinal organoids that have been transformed into colorectal carcinoma utilizing genetic engineering [179]. This preclinical model can be used to understand the role of low-frequency mutations by helping delineate those that are truly necessary for tumorigenesis versus those that are just passenger mutations. Having an understanding of the low-frequency mutations that confer survival to PDA tumor cells can then be exploited for targeted drug-development. Ongoing work (including work from JRB's laboratory) will validate the significance of this model for the pancreatic cancer research community and for the promise of precision therapy.

Beyond Genetic Alterations: Finding Alternative Targets

Considering the genetic diversity that is observed in PDA, another option would be approaching the treatment of this devastating disease by utilizing novel therapeutic approaches. For example, in melanoma, the use of immunotherapy has revolutionized

the treatment paradigm and has resulted in impressive patient outcomes [180–182]. In patients with PDA, the treatment approach would most likely require a combination therapy, in part, due to the genetic diversity that PDA exhibits allowing for compensation to occur along another pathway with targeted blockade. Therefore, other innovative ways of delivering therapy to patients with PDA may be targeting key cellular processes in order to take advantage of a genetic vulnerability, such as the use of PARPi therapy. In this section, alternative strategies to provide "targeted" therapy in PDA in ways that are novel and go beyond genetic alterations that are obtained from tumor sequencing will be discussed.

Role of Posttranscriptional Modification

Synthesis of messenger RNAs (mRNAs) is one of the essential functions of the cell. Once mRNAs undergo modifications in the nucleus, they are transported to the cytoplasm where they can be involved in a number of functions. Posttranscriptional gene regulation is a key cellular mechanism in which cells are able to modulate gene expression [183]. Regulatory mRNA elements can be present in any portion of the transcript (i.e., 5′-untranslated region (UTR), 3′-UTR, and in some instances even within the coding regions) [184, 185]. These regulatory elements lend themselves to regulation by RNA binding proteins (RBPs) and noncoding RNAs (i.e., microRNAs). Under nonpathologic conditions, posttranscriptional modification and regulation of gene expression are important in many cellular processes. However, there is also increasing evidence that posttranscriptional modification of mRNA transcripts plays an important role in tumor initiation and progression [186, 187]. In the following section, posttranscriptional modification by RBPs and how they can be used as predictors for aggressiveness, response to therapy, or potential therapeutic targets will be explored.

Role of RNA Binding Proteins in PDA Tumorigenesis

RNA-binding proteins (RBPs) are master regulators of mRNA processing and play a role in many vital cellular functions [188]. In cancer, RBPs play a powerful role in driving tumorigenesis, as they are expressed at high frequencies [189].

Perhaps one of the most well-studied RBP is Human Antigen R (HuR), also known as embryonic lethal, abnormal vision, and Drosophila-like 1 (ELAVL1) [190]. HuR is primarily expressed in the nucleus; however, upon exposure to stress, such as nutrient deprivation, hypoxia, or DNA damage, HuR translocases to the cytoplasm. HuR coordinates a pro-survival network of gene expression by binding to mRNA targets that support cell-survival functions [191, 192]. In vitro, silencing of HuR has been shown to result in decreased tumor growth, impaired migration and invasion, and anchorage-independent growth [193]. Moreover, a number of studies have also demonstrated downstream pro-survival targets of HuR that are important in tumorigenesis [8, 194–196]. Finally, a CRISPR knock-out model of HuR in PDA has demonstrated a unique xenograft lethal phenotype in PDA tumor cells [197].

HuR has been shown to be important as both a therapeutic target and a potential biomarker in PDA. Small molecule inhibitors of HuR have been used both in vivo and in vitro [8, 198, 199].

Targeting of HuR by small molecule compounds or siRNA nanoparticle strategies have shown great promise; and there is a hope that these strategies will make it into early phase human trials within the next few years. To date, HuR has also been extensively studied as a biomarker in PDA. In one study, patients with high cytoplasmic HuR have been associated with higher T-stage [200]. And a subsequent study showed that in patients with high cytoplasmic HuR, 5-FU-based therapy as associated with longer disease-free survival when compared to gemcitabine treatment [201]. Additional studies are needed to further elucidate the utility and role of HuR as a biomarker in patients with PDA. HuR may also represent another therapeutic option in PDA, as a drug sensitizer, in order to target a critical drug resistant network in PDA cells, especially in the tumor microenvironment where cells are exposed to low glucose, hypoxic conditions.

Epigenetic Regulation and its Role in PDA

Epigenetic modifications of DNA, such as histone deacetylation (HDAC) or DNA methylation, have been implicated in tumorigenesis and in metastasis [202]. As an example, in PDA, inactivation of *CDKN2A* can often times occur due to methylation at its promoter [203]. And this concept, where silencing of TSGs occurs via epigenetic silencing, is not uncommon or unique to PDA. Moreover, epigenetic reprogramming and regulation have also been implicated in metabolic changes in metastatic lesions. This was evaluated in a study by McDonald and colleagues, where matched primary and metastatic samples of PDA were studied in 16 samples from 5 patients [204]. Interestingly, the genetic diversity between the primary tumors and metastatic lesions was unchanged, reaffirming the results from a prior study by Makohon-Moore and colleagues [31]. Yet cells present in metastatic samples had acquired and selectively maintained epigenetic control of a malignant gene expression phenotype in the absence of driver mutations that are metastasis specific.

Targeting of epigenetic regulation has been attempted in PDA with the use of HDAC inhibitors, such as vorinostat, which results in inhibition of tumor growth in vitro and in vivo [205–207]. Vorinostat has also been used in clinical trials (NCT00958688), where it was used in combination with 5-FU and radiation in patients with locally advanced PDA; however, the study has been terminated and there are no reported results. A DNA methyltransferase inhibitor, 5-azacytidine, has also been evaluated in patients with advanced PDA in combination with gemcitabine. This study has also been terminated.

Currently enrolling clinical trials that are targeting epigenetic regulation as a therapeutic strategy include a phase II clinical trial in which resected patients with node or margin positive disease who have completed adjuvant therapy go on CC-486 (oral azacytidine) (NCT01845805). Another utilizes decitabine and tetrahydrouridine in patients with mPDA who have failed other therapy (NCT02847000). Therapeutic

strategies that aim to target epigenetic modification/reprogramming may be a novel approach for targeted treatment in patients with PDA. This will most likely be further realized as our understanding of the role of epigenetic regulation in metastatic lesions expands, possibly lending itself as a viable therapeutic option in patients with advanced disease.

Multi-omic Profiling and Its Role in PDA

A new approach in biological analysis is one where data from multiple sources (e.g., omes) are utilized. This includes genomics, proteomics, epigenitome, transcriptome, etc., in order to study biomarkers and therapies [208]. Multi-omic profiling has been explored in PDA by the authors (MJP and JRB) in order to further delineate the relevance of genetic aberrations found in PDA [37, 209]. Multiple platforms exist in order to take advantage of multi-omic profiling, but at the present time, most utilize NGS. With the use of this strategy, phosphoproteomic data have been provided to clinicians successfully and used to guide therapy [38, 210].

Though still in its relative infancy in PDA, the approach to characterizing patients based on multi-omic profiling is powerful and holds a lot of promise. It also integrates a number of important aspects of the patient's tumor, such as its genetic composition and epigenetic modifications, and offers an opportunity to provide targeted therapy.

Dysregulation in Axon Guidance Pathways in PDA

Sequencing studies have revealed that in PDA there are aberrations in axon guidance pathways [19, 20]. Other studies have also found epigenetic regulation in *SLIT-ROBO, ITGA2*, and *MET*, members of the axon guidance pathway [211]. Under nonpathologic conditions, expression of genes in the axon guidance pathways is important in embryogenesis. However, in cancer, their aberrant expression has been linked to increasing the predisposition of tumor formation and progression [212–214]. The exact role of these factors in tumorigenesis is not yet elucidated in PDA and how it may contribute to cell migration, angiogenesis, and cell survival. Considering the degree of dysregulated expression that exists in this gene subset in PDA, additional studies are needed to further elucidate their role. However, these molecules may be potential effective targets in PDA and in other cancers in a personalized manner.

Targeting the Tumor Microenvironment

One of the hallmarks of PDAs is its pronounced desmoplastic reaction, which makes up the tumor microenvironment (TME), with a paucity of tumor epithelial cells [6]. As discussed before, in PDA, the tumor microenvironment has a very important role

in PDA tumorigenesis and has been shown to interact with the tumor epithelial cells resulting in tumor progression [215–217]. Cells that are associated with the tumor stroma include inflammatory, immune, mesenchymal, and endothelial cells [218]. Cancer-associated fibroblasts (CAFs), an example of mesenchymal cells, have also been shown to impose epigenetic and metabolic regulation of tumor epithelial cells [219]. Additionally, activated pancreatic stellate cells, which give rise to CAFs, play an important role in the deposition of extracellular matrix components and the production of cytokines and growth factors [220, 221].

A number of signaling pathways that are dysregulated have been found to be important in the maintenance of the tumor stroma and may be potential therapeutic targets in PDA. TGFβ signaling, as discussed above, is commonly dysregulated in PDA. Ligands produced by the tumor epithelial cells can result in activation of its signaling cascade in stroma cells due to paracrine action, which has been shown to lead to fibroblast proliferation [222, 223]. This interaction is what also makes the use of TGFβ inhibitors a promising therapeutic strategy in PDA. Hedgehog signaling, as discussed in the *Targeting NOTCH signaling in PDA tumorigenesis* section, is also another attractive targeted therapeutic strategy due to its role in the desmoplastic reaction that's common in PDA. The tumor stroma has also been evaluated as a prognostic marker. In a study by Bever and colleagues, the density and activity of the stroma was evaluated and high-stromal density was found to be associated with a longer disease-free survival [224]. Other studies have shown that undifferentiated PDA is associated with increased vascularity, raising the potential of VEGF inhibitors as another targeted therapy [225].

Considering the important role of the TME in PDA, especially as mediated by immune cells, a number of compounds have been used to target this specific interaction. In a multicenter, randomized, placebo-controlled, and double-blind clinical trial, ibrutinib, a Burton's tyrosine kinase (BTK) inhibitor, is being evaluated in combination with nab-paclitaxel and gemcitabine for patients with mPDA as a first line therapy (NCT02436668) [226]. Ibrutinib is also being evaluated in combination with durvalumab, a human IgG1 monoclonal antibody that binds PD-L1 and inhibits its interaction with CD80, in a phase Ib/II multicenter study in patients with relapsed or refractory mPDA (NCT02403271) [227]. Both of these studies have completed enrollment and are ongoing; however, no results have been published as of this writing.

Limitations to Precision Therapy in PDA

Molecular profiling has changed the approach to therapy in many cancers, including PDA. NGS and other novel technologies are now becoming routinely incorporated in the care of some patients. However, data on genetic analyses are only useful if patients can take advantage of targeted therapy, whether on- or off-label or in clinical trials, in addition to standard treatment.

It is evident that in PDA, there is a necessity to not only develop better therapies, but to also continue to expand our understanding of the genetic make-up of PDA.

Moreover, there is a need for technologies, such as NGS, to provide actionable data in a timely manner so that it becomes routinely incorporated in clinical practice. The results of the IMPaCT trial, which evaluated the feasibility of providing sequencing data to facilitate treatment with targeted therapy, underscore the need to move genomic and molecular information into routine clinical care in order to propel precision medicine as a standard of care treatment strategy in patients with PDA. This will require continued financial support of agencies behind clinical trials that embrace this approach.

With continued improvements in modeling systems, such as patient-derived organoids, there will be an increased understanding of the genetic and nongenetic landscapes of PDAs. The ability to capture the genetic variability that is present in each PDA in these model systems provides a unique research opportunity that could have a significant return in regards to patient treatment. The ultimate goal would be to recapitulate the genetic diversity seen in individual PDAs into the organoid models in an effort to further identify specific drivers of each PDA that will reveal optimal therapeutic opportunities. Model systems, such as the organoids, allow for drug screens, gene editing, and other manipulations that can improve our understanding of the significance of individual gene mutation events. For instance, at the present time, though a number of low-frequency mutations and pathways disrupted in PDA have been identified, the contribution that these mutations have to driving PDA or if they will be susceptible to the current arsenal of available therapies is not yet something that has been elucidated. Understanding the functional implications of these low-frequency pathway disruptions will be integral in guiding drug discovery and efficient clinical trial design. Ultimately, improvements in the preclinical models in PDA will be helpful to study the clinical relevance of targeting dysregulated pathways or genetic mutations and will most likely result in novel insights moving towards precision therapy for PDA.

Future Directions

Discoveries that underlie the genetic drivers in PDA have been identified in patient samples and established in GEMMs and ex vivo models. This has been incrementally translated into innovative, successful therapeutic approaches that hope to improve patient outcomes. Though at the present time there is a paucity of FDA approved targeted therapies for patients with PDA, the number of trials that are ongoing that utilize this approach is impressive. NGS has given researchers and clinicians an insight into the genetic diversity of PDA. This technology spans the spectrum – its utilization in research laboratories is increasingly becoming translated to use in the clinic. Though targeting of low-frequency mutations will most likely not yield a significant clinical benefit to many patients with PDA, it will hopefully result in an increased understanding of the tumorigenesis of this disease and, importantly, aid a subset of patients. In fact, the field has accepted that targeting 5–10% of patients at a time might be a logical approach to improving outcomes. This strategy has been descriptively termed as the "pie approach" to treating the disease (i.e., if about 10% of patients are matched with

the correct therapeutic strategy, it can lead to significant changes in overall patient outcomes). This can, in turn, be supported by next generation ex vivo models, which will lead to a better understanding of the PDA biology and hopefully will result in higher throughput of drug testing for each patient.

At the present time, in order to make meaningful impact in PDA, researchers, clinicians, and surgeons need to have realistic goals in order to change the current trend in PDA. Ultimately, surgical resection is the only therapeutic option that gives patients a chance for long-term survival. However, since only a minority of patients can benefit from surgery, there need to be improvements in screening, diagnostic, and therapeutic strategies in order to allow more patients an avenue to surgical resection. A schematic for a futuristic clinical trial that employs such a realistic goal for patients in the metastatic setting is presented in Fig. 2. At the time of diagnosis, all patients with metastatic disease should have tumor sampling of both the primary tumor and

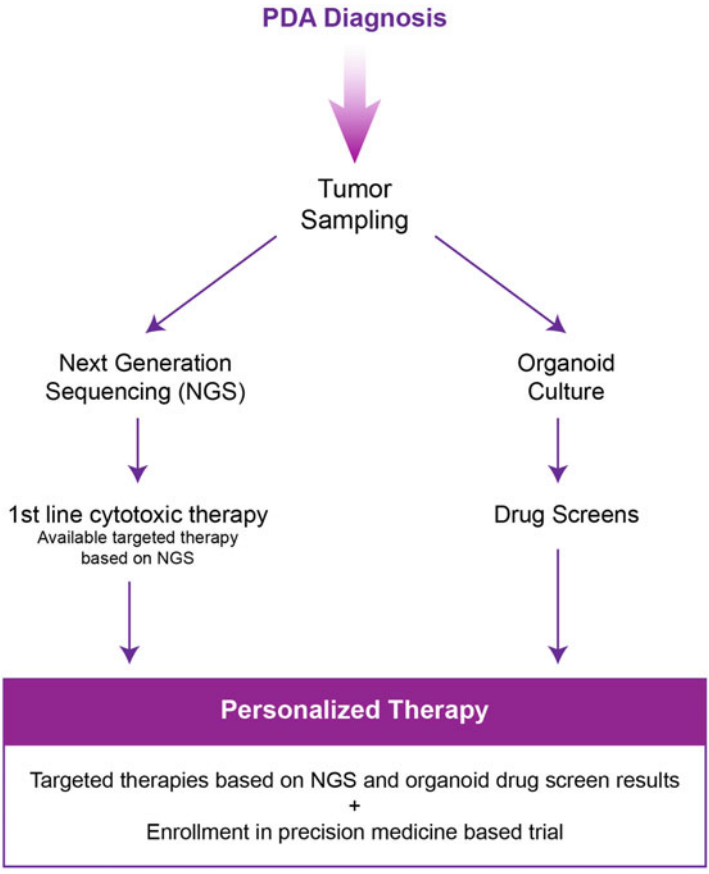

Fig. 2 Proposed clinical trial schema to optimize the use of precision medicine-based therapies

metastatic lesions. Both tumors should be sequenced and provided to a research laboratory for propagation into organoid cultures. The patient should be offered initial cytotoxic-based therapy followed by targeted therapy based on the sequencing results. Simultaneously, large-scale drug screens should be undertaken for both cytotoxic and targeted therapy while utilizing an organoid-like system. Based on these results from the preclinical model, patients should be advised which therapy they should pursue, whether on- or off-label, or as part of a clinical trial.

As a matter of fact, this similar approach can be employed in patients with all stages of PDA. For patients with resectable or locally advanced disease, tumor samples can be obtained, sequenced using whole-genome NGS, and propagated into organoid cultures. The goal should be to have sequencing and organoid drug screening results available to patients and clinicians in a clinically relevant timeline so that this information can be used for better informed clinical decision making. Moreover, utilizing preclinical data for predictive purposes (i.e., high cytoplasmic HuR and drug resistance) will allow clinicians to personalize the treatment approach to each patient. Ultimately, the power of combining NGS, preclinical modeling, such as organoids, and predictive markers will only be fully realized once the use of these technologies become validated.

Conclusion

The research community's understanding of the molecular drivers of PDA has increased over the past decade, with more and more studies delineating the genetic alterations found in this deadly disease. Despite these monumental strides, unlike in other cancers, this progress has been incremental, yet meaningful, in PDA. Our understanding of the implications of genetic aberrations, the role of the tumor microenvironment, metabolic alterations, epigenetic modification, and mechanisms of gene regulation in PDA will continue to increase. However, it is imperative that this is matched with equivalent progress of drug development that results in therapeutic options that can be used in the clinic. Maximizing the results of NGS will require aligning basic research with representative preclinical models. Ex vivo modeling that is done in parallel with NGS at the time of a patient's diagnosis will help support drug-screening that is based in the fundamental principles of targeted therapy. This strategy will also provide the backbone for well-designed clinical trials in order to produce results that lead to the realization of success in the domain of precision medicine (i.e., better treatment options and improved overall outcomes in patients with PDA).

Cross-References

- ► Animal Modeling of Pancreatitis-to-Cancer Progression
- ► Approaching Pancreatic Cancer Phenotypes via Metabolomics
- ► EGFR (ErbB) Signaling Pathways in Pancreatic Cancer Pathogenesis
- ► Emerging Therapeutic Targets in Pancreatic Adenocarcinoma

- ▶ Hedgehog Signaling Plays a Dual Role in Pancreatic Carcinogenesis
- ▶ Notch Signaling in Pancreatic Morphogenesis and Pancreatic Cancer Pathogenesis
- ▶ Smad4-TGF-β Signaling Pathways in Pancreatic Cancer Pathogenesis
- ▶ The Molecular Pathology of Precursor Lesions of Pancreatic Cancer

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Emerging Therapeutic Targets in Pancreatic Adenocarcinoma

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Contents

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Abstract

Pancreatic adenocarcinoma is one of the most lethal cancers but has limited therapeutic options necessitating continued investigation of new therapeutic agents. Recently, improved overall survival has been achieved with cytotoxic drug combinations including 5-fluorouracil, leucovorin, irinotecan, and oxaliplatin (FOLFIRINOX) and gemcitabine/nab-paclitaxel, but the success has been modest at best. More targeted approaches focusing on EGFR and MAPK signaling have also enjoyed marginal success. Accumulating evidence suggests that pancreatic tumors have increased dependence on metabolic pathways through both KRAS and KRAS-independent mechanisms and are broadly resistant to drug therapy due to stromal remodeling. Genetic and epigenetic vulnerabilities, such as inactivating aberrations in DNA damage repair, chromatin remodeling, and microRNA dysregulation, may reveal exploitable weaknesses. Modern approaches to drug development tailored to molecularly defined subsets of patients likely to respond to targeted therapies are needed to achieve more substantial progress in this disease in an era of precision medicine.

Keywords

Pancreatic cancer · Emerging therapeutics · Metabolic targets · DNA damage repair · Chromatin remodeling · Epithelial to mesenchymal transition · Stromal targeting · Pancreatic neuronal targeting · microRNA

Introduction

Pancreatic adenocarcinoma (PDAC) has very limited treatment options. The best benefit has been achieved with newer combinations of cytotoxic agents such as 5-fluorouracil, leucovorin, irinotecan, oxaliplatin (FOLFIRINOX) and gemcitabine/nab-paclitaxel, but with only modest overall survival benefits over single agent gemcitabine. Unfortunately, these therapies are associated with increased toxicity, and only the most functional patients can tolerate these regimens. Currently, the only targeted agent FDA approved for the treatment of PDAC is the epidermal growth factor receptor (EGFR) inhibitor erlotinib, which provides a minimal survival benefit measured in weeks. Identifying new therapies in pancreatic continues to be a major challenge. In this chapter, potential strategies for emerging therapeutics are discussed, including new approaches to KRAS signaling and suggested development of targets aimed at exploiting metabolic derangements, DNA damage repair, epigenetic modulation, and the tumor microenvironment.

MAPK Targeting

In pancreatic cancer, oncogenic KRAS serves as a necessary critical event in tumor initiation and growth maintenance [1]. Despite understanding the dependency of PDAC on the mitogen-activated protein kinase (MAPK) pathway through constitutive KRAS signaling, targeting RAS and its downstream effectors in PDAC has been unsuccessful. RAF and MEK inhibitors have largely been ineffective in KRAS-mutant tumors. In part, the failure of these agents has been thought due to redundancy in downstream signaling through MAPK (e.g., compensatory ERK reactivation) and the PI3K/mTOR pathways or loss of KRAS "addiction" with neoplastic progression [2].

Multiple mechanisms of resistance RAS and MEK inhibitors have been proposed. Treatment of KRAS-mutant tumors in particular has been challenging due to difficulty in selective targeting of the RAS GTPase, compensatory upregulation of ERK signaling, RAF dimerization, and formation of RAS-MEK complexes. Targeting of these mechanisms, likely in combination, may represent alternative approaches to circumventing poor responses to MEK inhibitors.

Lito et al. demonstrated that treatment of KRAS-mutant pancreatic cancer cells with MEK inhibitors resulted in a reduced ability to sustain prolonged ERK inhibition mediated through rebound ERK phosphorylation [3]. This rebound effect was found to be dependent on the release of feedback inhibition on the CRAF isoform. Two processes mediated rebound phosphorylation of ERK: relief of CRAF inhibition and formation of MEK-RAF kinase complexes. While activated ERK feedback typically inhibits RAF/MEK/ERK signaling by phosphorylating CRAF kinase, treatment with MEK inhibitors relieved the negative feedback signal by ERK on CRAF. Reactivation of CRAF resulted in downstream MEK phosphorylation and ERK rebound phosphorylation. In addition, MEK inhibitors induced complex formation of MEK with RAF kinases in KRAS-mutant cells but not BRAF-mutant cells. Increased association of MEK with RAF resulted in a subsequent increase in active phosphorylated MEK. This increased complex formation combined with increased active CRAF was shown to result in resistance to MEK inhibition. This study suggests that targeting the MAPK pathway will require rationally designed small molecule inhibitors or antibodies to block MEK-RAS complex formation while also inhibiting CRAF kinase activity.

Metabolic Pathways

Metabolic adaptation to changing environmental conditions is a critical component of tumorigenesis. In 1924, Otto Warburg described the ability of tumors to generate adenosine 5′-triphosphate (ATP) by fermentative metabolism through glycolysis, even in the presence of oxygen, rather than prioritization through mitochondrial oxidative pathways as occurs in normal cell metabolism. This seemingly inefficient method of ATP production is thought to benefit cancer cells since glucose and glutamine can be shunted toward synthesis of the necessary building blocks to

maintain rapid cell growth and division [4]. Glucose and glutamine can be diverted into pathways for production of the macromolecular precursors for the synthesis of fatty acids (e.g., acetyl-CoA), nonessential amino acids, and nucleotides (e.g. ribose) (Fig. 1).

In most mammalian cells, since nutrient supply is not typically restricted, nutrient uptake and utilization is tightly controlled to prevent excessive proliferation unless a growth factor is present to stimulate cell growth and division. Through the acquisition of oncogenic mutations, cancer cells circumvent growth factor dependence by altering signaling pathways to promote cell growth and survival. Key to the maintenance of proliferative capacity, the "Warburg effect" has now been linked to a number of oncogenic pathways, including KRAS, AKT, and MYC, and glucose deprivation itself has been implicated as a driving force in the acquisition of KRAS mutations [5].

Recently, a renewed interest in exploiting metabolic pathways in pancreatic cancer has been seen based on advances in transcriptome and metabolomic research. The impact of KRAS in exerting control over numerous metabolic pathways has been shown in a number of studies to be critical in carcinogenesis and maintenance of pancreatic cancer, thus presenting potential opportunities for therapeutic targeting. Metabolic pathways that have been implicated in KRAS-mediated tumorigenesis include glycolysis, the pentose phosphate pathway (PPP), and the tricarboxylic acid (TCA) cycle [1].

The impact of metabolic reprogramming on tumorigenesis is particularly intriguing given evidence that PDAC tumor cell lines were shown to exhibit distinct metabolic profiles [6]. Metabolomic and transcriptomic analysis of 38 pancreatic cancer cell lines identified three metabolic subtypes: slow proliferating, glycolytic, and lipogenic. Glucose and glutamine dependence and utilization either favored growth through glycolytic or lipid synthesis pathways leading to specific metabolic vulnerabilities that could be potentially exploited with pathway-specific metabolic inhibitors. Interestingly, even within the confines of in vitro experiments, some tumor cell lines also exhibited the ability to switch phenotypes based on metabolic stressors and highlight the difficulty in stratifying tumors into a one-size-fits-all therapeutic strategy.

The dependency of PDAC maintenance on KRAS-mediated metabolic changes was eloquently demonstrated in a pancreas-specific doxycycline-inducible $KRAS^{G12D}$ transgenic murine model, dubbed the *iKras* model [1]. As in the LSL-$KRAS^{G12D}$ model, this model required additional crossing with conditional *p53* knockout ($p53^{L}$) to a *iKras p53* mutant (*iKras* $p53^{L/+}$ or *iKras* $p53^{L/L}$) to recapitulate full malignant progression through invasive PDAC. Withdrawal of doxycycline induction resulted in extinction of KRAS expression and allowed analysis of transcriptional and metabolic changes with removal of oncogenic $KRAS^{G12D}$. Importantly, extinction of KRAS expression did not affect flux of glycolytic metabolites through the TCA cycle. The PI3K-AKT pathway and HIF1α also did not have significant impact on tumor metabolic signaling.

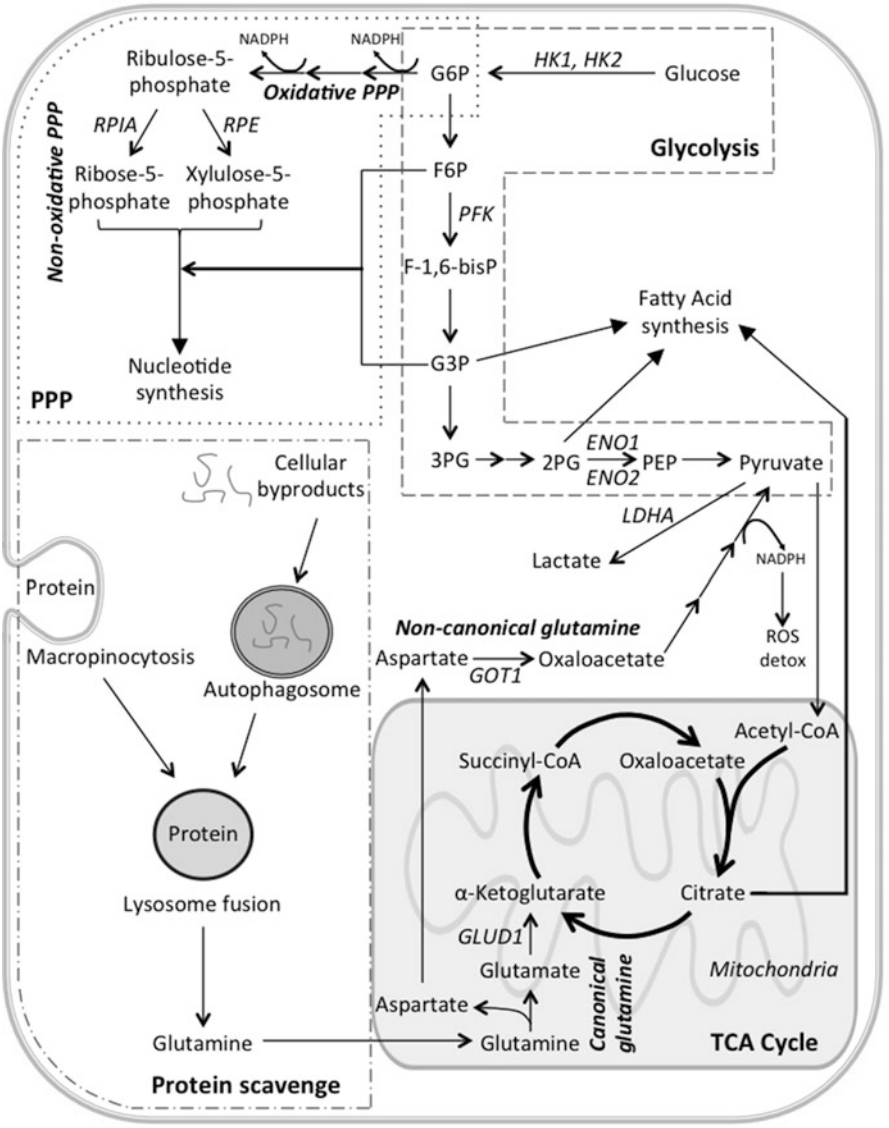

Fig. 1 Metabolic pathways involved in PDAC. *Abbreviations* (alphabetical order): *2PG* 2-phosphoglycerate, *3PG* 3-phosphoglycerate, *ENO1* enolase 1, *ENO2* enolase 2, *F-1,6-bisP* fructose-1,6-bisphosphonate, *F6P* fructose-6-phosphate, *G6P* glucose-6-phosphate, *GLUD1* glutamate dehydrogenase 1, *G3P* glyceraldehyde-3-phosphate, *GOT1* glutamic-oxaloacetic transaminase 1, *LDHA* lactate dehydrogenase A, *NADPH* nicotinamide adenine dinucleotide phosphate (reduced), *PPP* pentose phosphate pathway, *PEP* phosphoenolpyruvate, *PFK* phosphofructokinase, *RPE* ribulose-5-phosphate 3-epimerase, *RPIA* ribose-5-phosphate isomerase A, *ROS* reactive oxygen species, *TCA* tricarboxylic acid

Glycolysis

In line with the Warburg effect hypothesis, the i*Kras*/p53 study showed $KRAS^{G12D}$ was a major regulator of channeling glucose metabolites into synthesis of macromolecular molecules, including nucleotide and lipid synthesis pathways. KRAS itself appeared to drive glucose flux by upregulating uptake and shunting of glucose through glycolysis [1]. Glucose metabolic changes that were found to be dependent on $KRAS^{G12D}$ included regulation of multiple rate-limiting glycolytic enzymes (HK1, HK2, ENO1, and PFK1) and the glucose transporter GLUT1. These findings support the hypothesis that diversion of glycolytic intermediates into biosynthetic pathways promotes tumor proliferation and suggests PDAC reliance glycolytic processes can be exploited for therapeutic targeting.

KRAS-mediated regulation of ENO1, which regulates conversion of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate (PEP), may be of particular interest given enolases have been implicated in promoting tumor cell extracellular matrix degradation, migration, and proliferation [7]. In pancreatic cancer, ENO1 is upregulated, and expression promotes invasiveness and metastasis and mediates an immunoregulatory role in infiltrating T-cell effector function [8–10]. Interestingly, Daemen et al. identified ENO2 as one of the most differentially expressed genes in glycolytic cell lines compared to lipogenic cells [6]. This observation may lend support to a metabolomic approach to personalized treatment of pancreatic cancer by assessing tumor metabolic profiles with subsequent targeting of glycolytic enzymes, such as ENO1 or ENO2.

Lactic Dehydrogenase A

Also regulated by $KRAS^{G12D}$, lactic dehydrogenase A (LDHA) represents a particularly interesting target given its role in reduction-oxidation (redox) reactions with nicotinamide adenine dinucleotide (NAD) and NADH for both glycolytic and TCA cycles [11]. The two isoforms of LDH are expressed in tissue-specific distributions: LDHA in skeletal tissue and liver and LDHB in myocardium. Tumor tissue, including pancreatic adenocarcinoma, frequently demonstrates elevated levels of LDHA compared to normal tissue and has been implicated in tumor initiation, maintenance, progression, and aggressiveness and is associated with poor prognosis [11–13]. Increased metabolic demands of tumor growth and the hypoxic tumor environment drive up lactate levels. These processes have been shown to be regulated at the transcriptional and posttranslational level by c-MYC, HIF1α, and forkhead box protein M1 (FOXM1) [4, 14]. In pancreatic, lymphoma, and $KRAS^{G12D}$-driven lung mouse models, lactic dehydrogenase reduction delayed tumor xenograft progression due to increased oxidative stress from ROS production through enhanced pyruvate flux into the mitochondria. Such an approach may be potentially useful in PDAC tumors [12, 15].

Pentose Phosphate Pathway

Glucose flux into anabolic pathways for nucleotide synthesis was demonstrated to be significantly regulated by $KRAS^{G12D}$ with specific channeling of glucose metabolites through the non-oxidative arm of the pentose phosphate pathway (PPP) [1]. The PPP generates NADPH as well as ribose-5-phosphate for nucleotide synthesis. NADPH provides the reducing equivalents needed for macromolecular biosynthesis (e.g., fatty acids) and also relieves oxidative stress caused by mitochondrial ROS production, which is critical for KRAS-mediated tumorigenicity [16]. Comprised of the oxidative arm and the non-oxidative arm, this pathway is thought to be key to maintenance of the reducing equivalents needed for ROS detoxification.

The non-oxidative arm primarily generates ribose-5-phosphate (R5P) for DNA/RNA biosynthesis. RPIA and RPE, enzymes that regulate carbon metabolism in the non-oxidative PPP arm, were found to be significantly decreased with $KRAS^{G12D}$ extinction. The functional importance of this pathway in maintenance of PDAC was confirmed with knockdown of either or both RPIA and RPE, resulting in inhibition of xenograft tumor growth. These findings may suggest that KRAS-mutant PDAC have an exploitable reliance on this pathway to generate nucleotides for continued proliferation.

TCA and Glutamine Addiction

Consistent with the Warburg effect hypothesis, studies have also demonstrated that KRAS-driven oncogenesis requires glutamine diversion and catabolism into the TCA cycle for tumorigenesis [16, 17]. Fatty acid synthesis relies primarily on glutamine conversion in the mitochondrial tricarboxylic acid (TCA) cycle. Glutaminase, along with alanine aminotransferase, catabolizes glutamine to the alpha-ketoglutarate intermediate substrate of the TCA cycle. RAS-, MYC-, and AKT-dependent tumor cells have previously been suggested to require glutamine for mitochondrial metabolism [16].

Myc and Glutamine

MYC-overexpressing tumor cells have previously been shown to rely on glutamine for cellular proliferation. Promoter analysis and MYC knockdown studies suggest that MYC mediates $KRAS^{G12D}$-dependent transcriptional control of PDAC metabolic reprogramming [18]. As a byproduct of glutamine catabolism by the TCA cycle and further enhanced by oncogenic KRAS, mitochondrial reactive oxygen species have been shown to be potential regulators of cell cycle progression. This supports a distinct role for MYC as a regulator of metabolic intermediates primarily through control of glutamine entry into the mitochondrial TCA cycle. Given that a subset of PDAC tumors may have increased dependence on lipid synthesis pathways, blockade of glutamine diversion or generation of TCA cycle intermediates through MYC-mediated pathways may represent new avenues for therapeutic intervention.

Glutamine Scavenging

Targeting glutamine metabolism may be another novel approach to therapy and include specific enzyme targeting or inhibition of salvage of amino acids from proteins. PDAC tumors have been demonstrated to have increased reliance on a noncanonical glutamine metabolism. While normal cells typically utilize glutamate dehydrogenase 1 (GLUD1) to convert glutamate into α-ketoglutarate in the mitochondria, PDAC cells support the TCA cycle through glutamic-oxaloacetic transaminase1 (GOT1)-dependent conversion of aspartate to oxaloacetate, and ultimately pyruvate, through an oncogenic KRAS-mediated mechanism. Inhibition of enzymatic components of this pathway led to PDAC tumor growth inhibition through sensitization to ROS [17]. Other suggested mechanisms that support glutamine supply in PDAC also include glutamine from proteolytic degradation of extracellular protein, scavenged through an oncogenic RAS-mediated mechanism of macropinocytosis or through autophagy [19–21].

Methionine Salvage

The tumor suppressor gene CDKN2A on chromosome 9p21 is inactivated in more than 90% of pancreatic adenocarcinomas either through homozygous deletion, mutation, or hypermethylation [22, 23]. Deletion of CDKN2A occurs in roughly 40% of PDAC tumors. Because it is located about 100 kilobases telomeric from the CDKN2A gene, methylthioadenosine phosphorylase (MTAP) is frequently co-deleted with CDKN2A and is absent in 26–31% of pancreatic adenocarcinomas [23–25]. MTAP normally cleaves methylthioadenosine (MTA) to adenine and 5-methylthioribose-1-phosphate, which are essential for recycling AMP and generating methionine for adenine and methionine salvage pathways. In MTAP deficiency, neither adenine nor methionine can be salvaged. Cells are completely reliant on de novo purine synthesis for AMP production leading to sensitivity to inhibitors of de novo methionine synthesis and methionine starvation. Attempts at targeting MTAP-deficient tumors either through targeting of de novo synthesis or methionine depletion have been under investigation since the 1980s, but while successful in experimental models, a Phase II clinical trial aimed at inhibiting de novo methionine synthesis with *L*-alanosine was not successful [26, 27].

Recent analyses by Kryukov et al. and Mavrakis et al. demonstrated that MTAP loss resulted in a passenger vulnerability to protein arginine methyltransferase 5 (PRMT5) inhibition [23, 28]. Arginine methyltransferases transfer a methyl group from *S*-adenosylmethionine to arginine to produce methylarginine and *S*-adenosylhomocysteine (SAH). PRMT5 has been implicated in tumorigenesis through epigenetic regulation of cell cycle progression, promotion of EMT, and posttranslational modification of proteins, including p53 [29, 30]. In a pooled shRNA screen from 216 cancer cell lines, cell lines with loss of MTAP had marked differential expression of PRMT5 ($P = 1.64 \times 10^{-25}$) [23]. In in vitro and in vivo studies, MTAP-deficient pancreatic cancer cell lines with concomitant loss of CDKN2A and MTAP resulted

in markedly decreased tumor growth with PRMT5 knockdown that could be rescued with MTAP reconstitution. The same effect was not seen with CDKN2A deletion alone. MTAP passenger deletion, therefore, unmasked a dependence on PRMT5 in MTAP-deficient pancreatic cancer cells.

Importantly, the mechanism for PRMT5 sensitivity was found to be due to MTA accumulation from loss of MTAP [23, 28]. MTA competes with PRMT5 substrate *S*-adenosylmethionine (SAM). Increased MTA levels resulted in an MTA-bound form of PRMT5, whose catalytic domain became conformationally inactivated. Thus, the mechanism for sensitivity to PRMT5 inhibition is possibly due to impaired methyl group transfer functions downstream from PRMT5. In a separate study, MTAP-deficient tumors were also sensitive to knockdown of methionine adenosyltransferase II alpha (MAT2A), which converts ATP and methionine into SAM. MTAP-deficient cells were also sensitive to loss of PRMT5 and the PRMT5 co-complex factor RIO kinase 1 (RIOK1) [31]. Upstream and downstream mediators of PRMT5 signaling in methionine metabolism could therefore suggest exploitable vulnerabilities in the subset of pancreatic cancers with co-deleted CDKN2A/MTAP. The significance of PRMT5 with regard to chromatin remodeling should also be noted (see section "Chromatin Remodeling").

DNA Repair Genes

The DNA damage response (DDR) can play seemingly opposing roles when it is defective or intact, depending on the cellular context. A defective DNA damage response in tumor cells promotes genomic instability and tumorigenesis, but conversely, increased signaling of an intact DDR may also enhance resistance to therapies by repairing the induced genotoxic stress and preventing cell death [32]. The most common inherited genetic aberration in familial pancreatic cancers is BRCA1 and BRCA2 germline mutations, accounting for up to 17% of patients [33]. The presence of BRCA1/BRCA2 mutations increases the risk of pancreatic cancer up to sevenfold compared to the general population [34]. BRCA pathway mutational signatures are the most frequently identified genes associated with genomically unstable PDAC tumors [35, 36]. Of these BRCA pathway (BRCA1/2, ATM, or PALB2; Fig. 2) aberrations, 5% were germline and 12% were somatic mutations [36]. With deficiency of one component of the DDR, cancer cells may become reliant on other DNA repair mechanisms that would increase their susceptibility to additional DDR targeting, termed synthetic lethality. Stabilization of replication fork dynamics actually imparts a mechanism of chemoresistance in BRCA deficiency by rescuing cells from synthetic lethality. While PARP inhibitors in BRCA-associated pancreatic cancers are under investigation (NCT02042378, NCT02184195), other DNA repair defects may expand the therapeutic options to include combinations with radiation therapy [37]. Given that specific mutations in DDR pathways each represent a very small proportion of PDAC patients, this approach would likely require patient selection based on genetic analysis using a

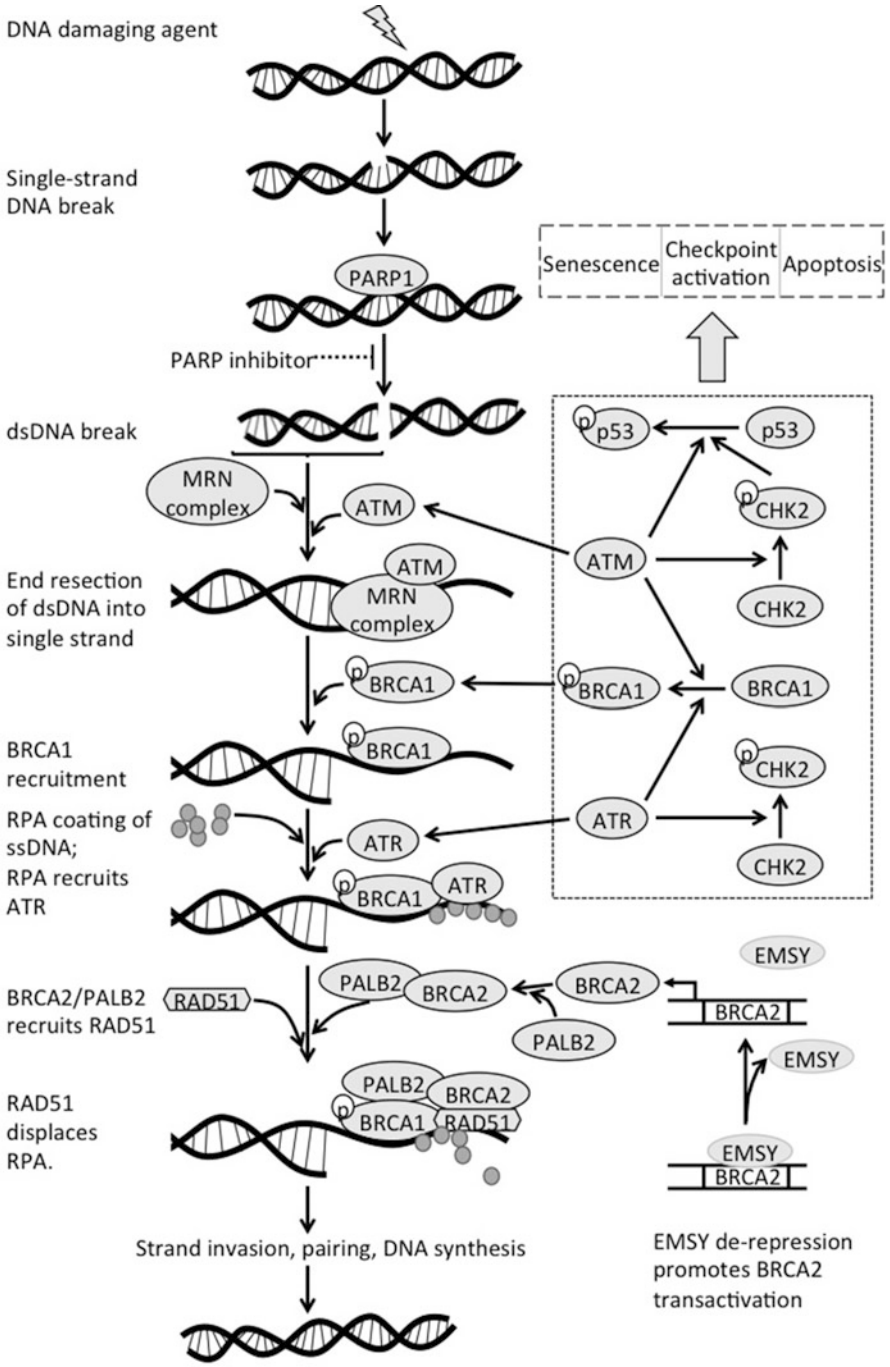

Fig. 2 (continued)

precision-medicine approach to enrich for pancreatic cancer subpopulations that may benefit. Below, potentially exploitable DDR genetic aberrations that may confer increased susceptibility to DNA damage repair inhibition are addressed.

PALB2

A nuclear binding partner of BRCA2, PALB2 facilitates BRCA2-mediated response by promoting its nuclear localization and stabilization for double-strand DNA (dsDNA) repair and homologous recombination. Disruption of PALB2's binding interaction with BRCA2 impairs repair of dsDNA breaks. Congruent with this, exomic sequencing analysis identified PALB2 as a pancreatic susceptibility gene in familial pancreatic cancer [38]. Germline deletions as well as truncating PALB2 mutations have been found in 3–4% of familial cases of pancreatic cancer [35, 36, 38, 39]. Included among these genetic alterations, a 6.7 kb germline deletion of PALB2 of exons 12 and 13 would notably interfere with PALB2's binding domain to BRCA2 [39].

ATM

Found on chromosome 11q, ATM is a serine/threonine kinase involved in the repair of double-strand DNA breaks as well as integration of signaling networks in response to genotoxic stress and cellular homeostasis. ATM aberrations are one of the most common genetic alterations and have been identified in up to 8% of pancreatic cancers [40, 41]. An immunohistochemical analysis of 57 patients also identified low ATM protein expression in 66% of pancreatic tumor samples compared to 8% of normal pancreatic tissues with decreased protein expression correlating to a more aggressive PDAC phenotype (less differentiated, more nodal metastases) [42]. In support of this, PDAC $ATM^{-/-}$, $KRAS^{G12D/+}$, and $p48^{Cre/+}$murine models suggest that ATM loss results in increased EMT and cancer cell stemness to promote PDAC progression and aggressiveness that further enhanced the effects of oncogenic KRAS. ATM loss additionally contributes to promotion of ROS production [43].

The synthetic lethal approach may also be effective in ATM-deficient PDAC tumors. Given that PARP inhibitors have shown potential for efficacy for prostate cancer, they may also hold promise in ATM-mutant pancreatic tumors [44]. Inhibition of the CHK1 pathway either through upstream ATR (ataxia telangiectasia and

Fig. 2 Homologous recombination DNA repair. *Abbreviations* (alphabetical order): *ATM* ataxia-telangiectasia mutated, *ATR* ataxia-telangiectasia and Rad3-related protein, *BRCA1* breast cancer 1, *BRCA2* breast cancer 2, *CHK1* checkpoint kinase 1, *CHK2* checkpoint kinase 2, *EMSY* BRCA2-interacting transcriptional repressor, *MRN complex* Mre11-Rad50-Nbs1, *PALB2* partner and localizer of BRCA2, *RAD51* RAD51 recombinase

Rad3 related) inhibition or direct CHK1 blockade may also be effective in ATM-deficient cancers. Preclinical evidence suggests that blockade of this pathway in multiple tumor types, including pancreatic cancer, may sensitize cells to DNA-damaging chemotherapy or radiation [45]. Direct small molecule inhibitors of ATM and ATR are under development for treatment of tumors with DDR deficiencies [46].

BUB1B

BUB1B (budding uninhibited by benzimidazoles) is a kinetochore protein that is critical to the mitotic spindle checkpoint that is activated by ATM and has been associated with genomic instability [47]. Less frequent than BRCA, PALB2, or ATM alterations, deleterious mutations in BUB1B were found in 1 of 39 familial pancreatic cancer patients [40]. However, bioinformatic analysis based on function and protein-protein networks suggested that BUB1B was one of the most differentially expressed gene hubs compared to normal pancreas [48]. Interestingly, PDAC tumors that bypassed KRAS dependency through a YAP1-dependent mechanism also consistently upregulated BUB1B suggesting it deserves further investigation as a possible mechanism for KRAS escape (see section "Epithelial to Mesenchymal Transition (EMT)") [49].

Chromatin Remodeling

Rapid modulation of gene expression is regulated at multiple levels beyond the simple gene coding sequence. Variable gene expression is further defined by cooperative epigenetic mechanisms such as histone modification, DNA methylation, and microRNAs to control cellular functions. Deregulation of epigenetic phenomena leads to aberrant signaling promoting PDAC development and progression.

SWI/SNF Complex

Multiple inactivating mutations in chromatin remodeling have been identified through genomic analysis [35]. Genetic mutations have been reported in PDAC in up to 14–34% of genes encoding components of the SWItch/sucrose non-fermentable (SWI/SNF) multiprotein complex, which modulates transcription by disrupting the DNA-to-histone contact in the nucleosome through ATP hydrolysis [36, 50]. The SWI/SNF complex is comprised of 15 subunits in various combinatorial assemblies. Mutations in the various subunits of the complex have been identified with clear tumor suppressor function [50, 51].

Encoding the BAF250a subunit of the SWI/SNF complex, ARID1A represents one of the most commonly mutated genes involved in chromatin remodeling and is a marker of poor prognosis in PDAC [52]. Depletion of ARID1B decreased the

viability of ARID1A-deficient pancreatic cancer cells compared to ARID1A-proficient cells, suggesting that a synthetic lethal approach to ARID1A-mutant PDAC tumors may be exploited [52, 53].

In a study by Shain et al., ARID1B deletions were found in 77% of 48 patient-derived xenografts and 22 PDAC cell lines [50]. Fifty-two of these deletions were single copy deletions indicating that typically only one subunit component of the complex would be affected. Two cell lines (PANC1 and MIAPaCa2) had loss at two separate subunits. Notably, on an individual level, each subunit harbored inactivating gene alterations in 2–10% of samples. Functional studies have suggested a possible tumor suppressive role for components of the complex. While promising, given the combinatorial complexity of the SWI/SNF complex, the impact of the stoichiometric distribution of subunits with loss of individual or multiple subunits on oncogenesis is likely to be complex and will require further investigation to develop viable therapeutic options [50].

KMD6A

Inactivating genetic aberrations of the histone demethylase KMD6A has been found in 18% of pancreatic cancers [35]. As a negative modulator of DICER transcription, KMD6A inhibition promotes EMT transformation through miR-200-mediated derepression of zinc finger E-box binding homeobox 1 (ZEB1) expression [54]. Limited preclinical data is available in pancreatic cancer. Significantly though, a relationship between KMD6A may exist with SWI/SNF complexes, PRMT5, and PRC2 (polycomb repressive complex 2). PRC2's catalytic subunit EZH2 (enhancer of zeste homolog 2) is a methylating enzyme known to have pro-oncogenic function in multiple tumor types, including PDAC, and EZH2 inhibitors are currently under Phase I investigation (NCT01897571, NCT02082977) [55–57].

SWI/SNF complexes have been found in association with PRMT5 (see section "Metabolic Pathways") and PRC2 to downregulate tumor suppressor gene transcription of suppressor of tumorigenicity 7 (ST7), nonmetastatic 23 (NM23), retinoblastoma-like protein 2 (RBL2) message [58]. Bromodomain protein 7 (BRD7), a SWI/SNF-associated protein that recognizes acetylated histones, recruits not only PRMT5 and PRC2 but also KMD6Ato the same tumor suppressor gene sites as PRMT5 and PRC2 [59]. KMD6A acts as an activating transcriptional regulator, suggesting a counter-regulatory role for KMD6A to PRMT5 and PRC2.

With loss of KMD6A in a significant proportion of PDAC tumors, PRMT5 and PRC2 hypermethylation could conceivably be unchecked in KMD6A-deficient PDAC tumors that leads to repressed expression of multiple tumor suppressor genes. Enriched selection of PDAC patients who have deficiency of KMD6A in combination with CDKN2A/MTAP co-deletion and/or EZH2 overexpression may identify patients who may derive added benefit from broader derepression of tumor suppressor genes through treatment with inhibitors of PRMT5, EZH2, BRD7, histone deacetylases, or methyltransferases.

Transcription Factors

EMSY

EMSY encodes a protein that abrogates BRCA2-mediated effects through transcriptional repression of BRCA2 and disruption of BRCA2 binding to RAD51, a protein interaction critical to homologous recombination repair of double-strand DNA breaks. In sporadic pancreatic cancers, EMSY gene amplification was found in 13.6% (8 of 59) tumor samples and mRNA overexpressed in 45% (9 of 20) pancreatic cancer cell lines [60]. Increased expression of EMSY results in inefficient homologous recombination repair of double-strand breaks and genomic instability, simulating BRCA2, PALB2, or ATM deficiency. Most current strategies that induce synthetic lethality rely on loss of DDR mechanisms. As an alternative approach, directly inhibiting pathways whose expression mimics deficient DDR mechanisms may represent a broader synthetic lethality approach. EMSY has also been implicated in chromatin remodeling through protein complex formations and promoter regulation of histone lysine demethylase KDM5A and the histone deacetylases HDAC1 and HDAC2 [61]. The role of EMSY in epigenetic modulation is particularly interesting given the expression of HDACs and the high frequency of hypermethylated genes in PDAC [62, 63]. Given EMSY's frequent gene amplification and overexpression in PDAC, its roles in mimicking BRCA2 deficiency and modulating epigenetic regulation, strategies to directly inhibit EMSY, or its downstream effectors may represent viable avenues for therapeutic intervention [62].

The p63 Family

Recent evidence suggests that the p63 family may be involved in DNA damage repair and EMT to increase tumorigenesis, metastatic potential, and chemoresistance and thus may be of therapeutic interest [64]. The p63 family additionally interacts with Wnt, mTOR, Notch, and sonic hedgehog pathways [64–66]. Unlike its p53 homologue, which is mutated in 60–70% of PDAC, p63 is rarely mutated. However, genetic loci variants have been linked to increased risk of pancreatic adenocarcinoma [67]. While the TP63 gene encodes for multiple isoforms, the two main variants TP63ΔN and TAp63 have opposing effects to regulate cellular function. The full-length transactivating isoform, TAp63, is transcribed from the promoter upstream from exon 1 of chromosome 3p27 and mediates tumor suppressor effects. In contrast, TP63ΔN (NP63) is pro-oncogenic and is transcribed from an alternate promoter in intron 3 as a truncated isoform missing the N-terminal transactivation domain.

The TAp63 isoform acts as a tumor suppressor by inducing cellular senescence and inhibiting metastasis through transcriptional activation of microRNA processing enzyme DICER1 and microRNA miR-130B [68]. Loss of TAp63 also results in defective fatty acid oxidation, mitochondrial function, and glucose uptake, making it particularly interesting in the context of the significant metabolic derangements found in PDAC tumors (see section "Metabolic Pathways") [69]. Countering the

function of TAp63, TP63ΔN acts as dominant negative for TAp63 as well as for p53 and p73 by competing for promoter elements or by direct protein inhibition [64]. TP63ΔN and its transcriptional targets were found to be highly expressed in the more aggressive squamous PDAC subtype compared to other subtypes [36]. In pancreatic cancer cell lines, TP63ΔN was the dominant isoform, exerting transcriptional control over EGFR with downstream upregulation of ERK, AKT, and JNK (c-Jun N-terminal kinase) signaling to promote proliferation, migration, and invasion [70]. The 14-3-3σ promoter was also activated by TP63ΔN to increase resistance to cisplatin-induced apoptosis. Although it is likely a balance of TAp63 versus TP63ΔN that directs tumorigenic potential, disruption of TP63ΔN pro-tumorigenic effect may be of particular interest as a therapeutic target.

Epithelial to Mesenchymal Transition (EMT)

Epithelial cells that acquire a more mobile mesenchymal phenotype have increased capacity to migrate, invade, and disseminate systemically in a process of developmental plasticity called epithelial to mesenchymal transition (EMT). Loss of apical-basal polarity and disruption of tumor cell adhesion are modulated by E-cadherin, Twist upregulation, and ZEB1 that are largely coordinated by cross talk among TGFβ, Wnt, and Notch pathways along with miRNAs [71, 72]. TGFβ signaling promotes Snail and ZEB1 expression that, in turn, appears to control a feed-forward mechanism of transcriptional suppression of the pro-epithelial microRNA-200 family [73].

In pancreatic adenocarcinoma, EMT is thought to play a key co-regulatory role with cancer-associated fibroblasts in remodeling stroma. Pancreatic cancer-associated EMT is activated and maintained through TGFβ/TNFα signaling that is associated with sustained activation of RAS/MEK/ERK signaling [74]. Evidence suggests that the relationship between KRAS and epithelial to mesenchymal differentiation may present a therapeutic window for targeting.

KRAS Addiction and EMT

KRAS dependence or "addiction," where tumor cell growth is reliant on continued KRAS signaling, represents a potentially exploitable pathway. A prior study by Singh et al. suggested a link between EMT induction and loss of KRAS dependency [75]. However, KRAS-addicted cells remained sensitive to inhibition of SYK and RON kinases as well as integrin-beta6 with distinctly reduced tumor cell growth and increased caspase-3-mediated apoptosis. Similar inhibitory effects were not seen in KRAS-independent cell lines. Inhibition of SYK, RON kinase, or integrin-beta6 may offer benefit in selected patients with PDAC tumors selected for KRAS dependency.

KRAS Independence and EMT

Although KRAS has been established to play essential roles in initiation and maintenance of PDAC tumors, loss of KRAS addiction allows tumor escape and development of resistance mechanisms that make targeting KRAS signaling more difficult.

Strong evidence supports a role for YAP1 mediating KRAS-independent growth to bypass KRAS dependence. Yes-associated protein 1 (YAP1) is a transcriptional coactivator involved in regulating pancreas development, DNA replication, cell cycle progression with pro-tumorigenic roles in the Hippo kinase cascade, and β-catenin/Wnt signaling [76, 77]. In two key studies, tumorigenic growth from loss of KRAS was rescued by reciprocal YAP1 gain of function [49, 78]. Utilizing the previously described doxy-inducible *iKras* PDAC model, tumor relapse was observed in mice despite extinction of KRAS expression suggesting that KRAS was no longer necessary for tumor growth [1, 49]. In relapsed tumors, PDAC maintenance circumvented dependence on KRAS signaling through acquisition of YAP1 gene amplifications and downstream binding to TEA domain family member 2 (TEAD2) to activate transcription of cell cycle and antiapoptotic genes. In the second study by Shao et al., YAP1 expression rescued previously KRAS-dependent lung cell lines whose KRAS expression was suppressed. Transcriptional activation was found to be mediated through a TEAD-independent mechanism through FOS-mediated transcriptional control of the EMT program. KRAS-independent cells were noted to be enriched with an EMT signature. These two studies suggest that YAP1 represents a potential targetable KRAS resistance mechanism in PDAC tumors.

Stroma

PDAC tumors prominently demonstrate a strong desmoplastic reaction leading to development of a peri-tumoral fibrotic stroma. An increasing volume of literature supports a role for stromal signaling in modulating tumor carcinogenesis, growth, immunosuppression, and chemoresistance [79]. PDAC stroma is composed of mainly dense extracellular matrix (ECM) proteins, activated pancreatic stellate cells (PSCs), cancer-associated fibroblasts (CAFs), and immune cell infiltrates. Under pro-inflammatory conditions of injury and carcinogenesis, autocrine and paracrine cytokine and growth factor signaling in concert with tumor cells activate PSCs and CAFs to secrete ECM components, including collagens, integrins, and fibronectin to form the fibrotic matrix.

The nature of the stroma-tumor interaction is under some debate since studies have conflicted on whether the stroma protects versus inhibits tumors. Early evidence suggested that stroma acted as a physical barrier limiting drug delivery to tumor cells through sonic hedgehog (SHH) signaling or through vascular collapse from increased interstitial fluid pressures [79, 80]. The seminal paper by Olive et al. showed that ablation of stromal CAFs through SHH inhibitor IPI-926 (saridegib) in transgenic KPC mouse model allowed increased intratumor vessel growth and gemcitabine penetration [80]. Follow-up clinical trials showed no benefit and possibly even detriment with sonic hedgehog inhibitors [79]. Recent preclinical studies

have suggested that the stroma actually serves to constrain pancreatic tumor growth and depletion of stroma enables accelerated PDAC growth.

Subsequent strategies to modulate stromal interactions have included targeting of acellular extracellular matrix components in addition to recent literature indicating a significant role for microRNAs. MicroRNAs are discussed in section "MicroRNAs (miRNAs)" of this chapter. Far from being a bystander to cellular signaling, the acellular stromal elements have also been shown to promote carcinogenesis, stromal remodeling, metastasis, chemoresistance, and immunosuppression.

Enzymatic depletion of the matrix polysaccharide hyaluronic acid (HA) demonstrated improved tumor perfusion by decreasing interstitial pressures, increased vascular permeability, and microvascular re-expansion that allowed increased gemcitabine delivery with decreased tumor growth and improved survival [79]. Early phase I/II trials with enzymatic HA depletion with recombinant PH20 hyaluronidase (PEGPH20) demonstrated good tolerability and suggested potential benefit in high HA-expressing tumors. PEGPH20 is now in a randomized, double-blind, placebo-controlled phase III trial with nab-paclitaxel and gemcitabine and in phase I/II trials with other drug combinations (NCT02715804, NCT02241187, NCT01959139).

Additional approaches to depletion of various matrix components may be of benefit. One such strategy may be to target proteins that may be dependent on the metabolic derangements found in PDAC tumors. Pancreatic tumors are known to acquire *O*-linked glycosylation patterns with malignant progression [81]. As are characteristic of adenocarcinomas, mucins are highly expressed on epithelial cell surfaces whose core proteins are also heavily post-translationally modified in both normal and cancer tissue. In pancreatic cancer, mucins MUC-1 and MUC-4 are differentially glycosylated, likely by polypeptide glycosyl transferases, to produce glycoforms that act as tumor-associated carbohydrate antigens (TACA). They contain truncated glycan structures with sialyl Tn (STn, NeuAcα2-6GalNAc) and Tn (GalNAc) antigens that are not found in normal pancreas and are increased with metastatic liver disease [81, 82].

Galectins belong to a family of lectins with a carbohydrate recognition domain that binds extracellular or ECM glycans, such as MUCs, specifically at the N-acetyllactosamine (Galβ(1–4)-GlcNac; LAc-NAc) units [83]. Galectin-1 (GAL1) and galectin-3 (GAL3) are aberrantly overexpressed in epithelial cells and stroma of pancreatic tumors and have well-documented roles in tumorigenesis, migration, invasion, and immunosuppression [8, 83, 84]. Oncogenic RAS signaling appeared to be activated by galectin-3 through direct KRAS binding in an orthotopic PDAC mouse model [85]. Galectins, particularly galectin-1, have been implicated as playing significant roles in mediating tumor-stromal interactions. GAL1 promotes stromal activation and acinar-to-ductal metaplasia through a SHH-dependent mechanism to promote progression and invasion [86]. Its role in immunosuppression was also supported by Gal1 knockout in a transgenic PDAC (*Ela-myc*:Gal1$^{-/-}$) mouse model, where effector immune infiltration was increased and desmoplasia notably was decreased [86, 87].

Pancreatic Neuronal Targeting

The pancreas is richly innervated with a complex network of both extrinsic and intrinsic neural inputs derived during embryonic development from the primitive foregut. Sensory information from the digestive system is conveyed to the central nervous system via an extrinsic system of autonomic afferents mainly distributed along the vagus nerves (parasympathetic) and splanchnic nerve trunks (sympathetic) through celiac and superior mesenteric artery plexi. Aggregates of neural cell bodies, called intrapancreatic ganglia, are distributed throughout parenchymal tissue and act as the intrinsic component of the pancreatic nerve supply.

Perineural invasion occurs in pancreatic adenocarcinoma with a reported incidence of up to 90–100% of PDAC cases and represents one of the most important prognostic factors for poorer survival [88, 89]. A mutual tropism between pancreatic tumor cells and neural tissue has been well documented. Histologic analysis of PDAC tumors has shown tumor cells spread continuously along nerve branches into the extra-pancreatic nerve plexus [90]. Tumor infiltration of neural tissue has been implicated as a major cause of regional recurrence after resection since innervation of lymph nodes provides a direct route for distant tumor cell dissemination through lymphatics.

Increasing evidence also indicates that nociceptive information mediates a reciprocal signaling interaction with neurotrophic factors to promote tumor growth and neural invasion. Preclinical models have consistently shown a role for neuronal modulation of inflammation in chronic pancreatitis, a known precursor to PDAC. Significant increases in immunoreactive neurotransmitters, such as calcitonin gene-related peptide (CGRP), substance P/tachykinins (SP/TK), neuropeptide Y (NPY), or vasoactive intestinal peptide (VIP), were demonstrated in the setting of chronic pancreatic inflammation. The neuropeptides CGRP and SP/TK in particular heavily co-localize with pancreatic nerves supplying pancreatic vasculature [91].

Alterations in neuronal growth factor protein expression, such as protein gene product 9.5 (pgp9.5), myelin P0 protein (MPP), nerve growth factor (NGF), TRKA, and p75, have been associated with glucose dysregulation in PDAC as well as increased perineural invasion [89]. Additionally, PDAC tumors demonstrate multiple aberrantly methylated promoters regulating neuronal growth and differentiation [62]. These data suggest that regulatory neuropeptide signaling plays a significant function in mediating PDAC neuronal invasion and that targeting these signaling pathways may alter the progression of pancreatic cancer.

ROBO/SLIT and Semaphorins

Integrated genomic analysis comparing a clinical cohort of 142 early-stage PDAC (clinical stages I to II) patients, KRAS mouse models, and cell line shRNA knockdowns showed frequent somatic aberrations of potential functional significance in axon signaling pathways [41]. Three axon guidance pathways were enriched: SLIT and roundabout (SLIT/ROBO) pathways, class 3 semaphorins, and ephrins. Up to

15% of patients had focal copy-number losses and 5% harbored mutations in SLIT2 and ROBO2. Amplification of class 3 semaphorins SEMA3A and SEMA3E was present in 18% of patients and mutations found in 3%. Corroborating patient molecular data, transgenic KRAS murine models of pancreatic carcinogenesis showed progressive mRNA expression changes in SLIT/ROBO and semaphorin pathways with transformation from normal pancreas to tumor.

The role of the SLIT/ROBO pathways in endothelial cell guidance is particularly interesting given the interdependent regulation of angiogenesis and neurogenesis [92]. SLIT proteins bind ROBO receptors to mediate repulsive cues in axon growth and inhibit cell migration of neurons as well as vascular sprouting and branching [93, 94]. SLIT2 mRNA expression has been shown to be decreased in PDAC cells. Additionally, restoration of the repellent axonal cues by SLIT2 inhibited unidirectional movement of PDAC tumor cells along contacted neurites [95]. These findings suggest that the absence of key negative regulators of neural migration and vascular growth may allow permissive invasion and dissemination of tumor cells along nerves and vessel tracts. Consistent with this, PDAC tumors also demonstrate epigenetically suppressed SLIT/ROBO pathway signaling through DNA hypermethylation [96]. As such, therapeutics aimed at ROBO/SLIT networks may limit PDAC progression and invasion.

MicroRNAs (miRNAs)

MicroRNAs are 19–25-nucleotide noncoding RNAs that regulate gene expression posttranscriptionally. They are transcribed by RNA polymerase II initially as precursor miRNAs, which are then processed into mature miRNAs by Drosha (nucleus) and Dicer (cytoplasm) [97]. The miRNA associates with Argonaute proteins to form the RNA-induced silencing complex (RISC). RISC binds target mRNAs and either blocks translation or initiates degradation of the target mRNA, as determined by complex interactions based on the degree of complementarity to the miRNA. A single miRNA may be able to bind a variety of mRNAs and vice versa, and consequently a single miRNA may affect the expression phenotype of multiple genes, and a single gene expression phenotype may be modified by multiple miRNAs. In cancers, miRNAs may block translation of oncogenes (tumor suppressor miRs) or tumor suppressor genes (onco-miRs). Conceptually, targeting of multiple miRNAs in combination may be possible treatment strategies to affect signaling at multiple regulatory levels by delivering tumor suppressive-miRs while inhibiting onco-miRs.

MicroRNAs act as critical modulators of PDAC pathogenesis, including carcinogenesis and stromal remodeling. At least 500 differentially expressed miRNAs have been identified in PDAC [98]. MicroRNAs with altered expression in pancreatic cancer compared to chronic pancreatitis, such as miR-217 and miR-196a, may offer targets to differentiate stromal changes from tumor. Key among these are miRNAs that interact to promote oncogenic pathways, such as KRAS and NF-κB, and mediate pro-tumorigenic processes such as EMT or stromal expansion.

Translation of KRAS itself is inhibited by multiple tumor suppressor miRNAs, including miR-217, miR-206, miR-145, and let-7 [97]. Let-7 miRNA is also regulated by oncogenic RAS and in PDAC its upregulation reverses EMT. Dysregulation of the miRNAs can lead to KRAS upregulation and layers of downstream miRNA signaling complexities to promote oncogenesis. Here, some of the more promising miRNAs documented in PDAC oncogenesis are discussed.

Tumor Suppressor miRs

miR-200 Family

The miR-200 family (miR-200a through c) promotes mesenchymal to epithelial transition (MET) to limit metastatic potential, invasion, and chemoresistance. Expression of miR-200 correlated with decreased EMT markers E-cadherin and Vimentin through targeting of ZEB1 and ZEB2 [72]. Their role in EMT regulation has implicated the miR-200 family in stromal remodeling as paracrine signaling agents that modulate cytokine signaling in the tumor microenvironment [99]. The miR-141 member of the miR-200 family is expressed at low levels in PDAC relative to normal pancreas tissue and has been associated with worse overall survival and negative clinical-pathologic characteristics such as tumor size, nodal status, and lymphatic invasion [100]. miR-141 inhibits YAP1, previously discussed in section "Transcription Factors" as a possible mediator of escape from KRAS addiction [78, 101]. Additionally, miR-141 has been implicated with roles in decreasing pancreatic tumor cell migration, invasion, and cell cycle progression [97].

miR-34

The miR-34 family is composed of three homologues (miR-34a through miR-34c) with variable tissue-specific expression. Their tumor-suppressive regulation of multiple critical pathways in PDAC oncogenesis suggests miR-34 delivery could be a potential therapeutic agent [97]. miR-34a and miR-34b inhibit tumor growth by inhibiting Bcl-2, Notch, and TGFβ signaling. Apoptosis may also be induced in PDAC cells through both p53-dependent and p53-independent functions. miR-34a additionally inhibited EMT, tumor cell proliferation, and cell cycle progression and reduced stem cell characteristics.

Onco-miRs

miR-155

Overexpressed in PDAC, upregulation of miR-155 is driven by activating KRAS mutations and is associated with poorer survival [102]. Its role in PDAC oncogenesis has been well studied [97]. Its function has been implicated in regulation of multiple signaling pathways key to PDAC pathogenesis, including signal transducer and activator of transcription 3 (STAT3), EGF, MAPK, NF-κB, IL-6, interferon-related

pathways, and inhibition of p53 activation. It has also been implicated in transforming pancreatic fibroblasts into cancer-associated fibroblasts.

miR-21

Signaling through KRAS also upregulates miR-21 expression [97]. In PDAC, miR-21 expression is increased and predicts worse outcome in node-negative disease. Expression of miR-21 correlates with chemoresistance, tumor cell growth, invasion, migration, proliferation, and recruitment of CAFs, as mediated through PI3K-AKT signaling, PTEN inhibition, and upregulation of antiapoptotic molecules, such as Bcl-2.

In Vivo Delivery of Small RNAs

While microRNAs (miRNAs) and other small RNAs such as siRNAs have promising characteristics for manipulation of PDAC signaling, current obstacles in their systemic delivery limit translation into therapeutic agents. In vivo delivery of small RNAs has been hampered by the inability to administer them systemically due to poor tissue penetration, cellular uptake, and rapid clearance due to rapid renal excretion and serum RNase degradation [103]. Naked nucleic acids cannot penetrate cell membranes through passive diffusion due to their hydrophilic nature, large molecular weight, and polyanionic charge and so require molecular modification to enter the cell. Delivery efficiency with intravenous administration has therefore been insufficient to achieve therapeutic benefit. Additionally, off-target gene silencing by siRNAs and immunogenicity of the siRNA duplex are considerations. These combined factors vastly limit the utility of small RNAs as systemic therapeutic agents despite clear in vitro efficacy. Efforts to increase their cell membrane penetration to reach cytoplasmic or nuclear targets and to prolong bioavailability have been heavily investigated. Attempts to improve systemic circulating half-life have focused on nanoparticle encapsulation of the nucleic acids to protect them from nuclease degradation and to improve cellular uptake. These approaches have included encapsulation in carrier systems, such as liposomes, or cationic complex formation with cationic lipids or polymers (e.g., polyethyleneimine [PEI], polyamide amine dendrimers [PAMAM]), but carrier-based strategies have been hindered by systemic toxicity although modifications such as polyethylene glycol (PEG) groups may ameliorate some effects.

Recently, tumor growth inhibition and improved survival were seen in a MYC-induced liver carcinoma mouse model with an intravenously administered dendrimer-encapsulated miRNA [104]. This approach was unique since the selection of the delivery vehicle was based on a novel methodological approach to identify candidate dendrimers from a chemically diversified library followed by progressive chemical modification through multiple in vitro and in vivo steps until a dendrimer was identified with the following features: high cellular penetrance, small RNA delivery efficiency, prolonged extracellular distribution (>6 days), and low toxicity

of the parent and degradation products. As a proof of principle, this degradable dendrimer GA2-SC8 was used to encapsulate tumor suppressor *let*-7g miRNA to form GA2-SC8 nanoparticles (NP) and intravenously injected on a weekly basis. Demonstrating high potency with a 13-fold increase in *let-7g* expression after 48 h, transgenic mice treated with GA2-SC8-*let*-7g NP demonstrated remarkably improved survival ($P = 0.004$) with minimal to no toxicity compared to their untreated and GA2-SC8-control counterparts. The lack of liver toxicity in this hepatocellular carcinoma model is of clinical importance given the high frequency of liver metastasis in PDAC. While it remains to be seen if the success of the GA2-SC8 dendrimer or the dendrimer selection methodology can be translated to effective small RNA delivery in humans, successful translation would open enormous opportunities for highly selective targeting with siRNA and miRNA.

Conclusion

Prior advances in treatment of pancreatic adenocarcinoma have seen modest success through empirical study of cytotoxic chemotherapy or radiation regimens rather than through targeted or rationally designed approaches. As technologies for multilevel genomic and expression-level analysis continue to advance, a refined approach to utilizing patient genetic information combined with PDAC pathway vulnerabilities will be critical to the discovery of new clinically useful therapeutics.

Key Research Points

- Novel pathways demonstrate exciting anticancer targets in preclinical studies.
- Metabolic reprogramming, stromal remodeling, and neuronal signaling pathways in pancreatic cancer offer promising targets for therapy.
- Treatments impacting chromatin remodeling and microRNAs present therapies aimed at altering signaling networks at the epigenetic level.
- Mutations in metabolic genes and DNA damage response may expose synthetic lethal vulnerabilities in pancreatic cancer.

Future Research Directions

- Recent multi-platform analysis of genetic and expression profiles for pancreatic cancer identify the heterogeneity of molecular drivers of disease. Further investigation of agents that exploit somatic mutational events in combination with metabolic vulnerabilities may offer strategies for tailored treatment of pancreatic cancers.
- Studies investigating agents to modulate the tumor microenvironment will inform development of novel treatment approaches and improve the distribution of investigational and standard therapeutic agents.

Clinical Implications

A personalized treatment approach to identifying individual tumor susceptibilities will be increasingly needed to address the heterogeneity in genetic and phenotypic features of pancreatic adenocarcinomas.

Cross-References

- ▶ Animal Modeling of Pancreatitis-to-Cancer Progression
- ▶ Apoptosis: Signaling Pathways in Pancreatic Cancer Pathogenesis
- ▶ Approaching Pancreatic Cancer Phenotypes via Metabolomics
- ▶ Chemotherapy for Advanced Pancreatic Cancer
- ▶ Developmental Molecular Biology of the Pancreas
- ▶ EGFR (ErbB) Signaling Pathways in Pancreatic Cancer Pathogenesis
- ▶ Epidemiology and Prospects for Prevention of Pancreatic Cancer
- ▶ Epigenetic Pharmacology
- ▶ Epigenetics and Its Applications to the Progression Model of Pancreatic Cancer
- ▶ Hedgehog Signaling Plays a Dual Role in Pancreatic Carcinogenesis
- ▶ Metabolism in Pancreatic Cancer
- ▶ Mouse Models of Pancreatic Exocrine Cancer
- ▶ Notch Signaling in Pancreatic Morphogenesis and Pancreatic Cancer Pathogenesis
- ▶ Pancreatic Cancer Stem Cells
- ▶ Pathologic Classification and Biological Behavior of Pancreatic Neoplasia
- ▶ Precision Medicine Based on Next-Generation Sequencing and Master Controllers
- ▶ Role of Tumor-Stromal Interactions in Pancreatic Cancer Invasion and Metastases
- ▶ Smad4-TGF-β Signaling Pathways in Pancreatic Cancer Pathogenesis

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