Genomic Alterations in Sporadic Pancreatic Cancer

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Abstract The prognosis for most patients afflicted by pancreatic cancer still remains dismal. With the majority of cases being diagnosed at advanced stages, only minimal improvements in survival rates have been achieved using current therapeutic approaches. Nonetheless, remarkable research efforts over the past decade have enabled a detailed understanding of the molecular mechanisms underlying the pathogenesis of pancreatic cancer. According to the current state of knowledge, pancreatic carcinogenesis is a multistep process that requires alterations in a compendium of oncogenes, tumor-suppressor genes and genome-maintenance genes. The most frequent aberrations (somatic point mutations and allelic losses) affect oncogenes (*KRAS2*) and tumor-suppressor genes (*CDKN2A/p16, TP53, SMAD4/DPC4*) that have a key role in transcription, proliferation and regulation of the cell cycle, amongst others. In addition to these known mutational "mountains," a wide number of less frequently altered genes ("hills") have been discovered, which play an important part in defining the unique biology and behavior of each individual pancreatic cancer. A deeper understanding of the genetic landscape of pancreatic cancer, enhanced by "next-generation" high-throughput technologies will hopefully promote the development of new methods for early diagnosis and facilitate improvements in current therapeutic approaches.

Introduction

Extensive clinical and research efforts have been conducted over the last few decades to improve the prognosis of patients with cancer. In some tumor types, such as breast and colorectal cancer, early detection and better therapeutic agents have

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led to a significant decline in mortality rates, even for advanced disease (Berry et al. 2005; Kopetz et al. 2009). Conversely, patients afflicted by pancreatic cancer still harbor a dismal prognosis, with mortality rates that approximate incidence rates (Siegel et al. 2012). Especially at advanced stages, prolonged survival is anecdotal, and although therapeutic regimens have recently shown promising results (Conroy et al. 2011), the overall prognosis remains dismal, underscoring our need for a more detailed molecular knowledge of this disease.

Genomic alterations that translate into gain or loss of function of critical genes represent a hallmark of cancer (Hanahan and Weinberg 2011), and pancreatic cancer is no exception. Molecular and epidemiological data support the importance of key genetic alterations in the pathogenesis of pancreatic cancer. For example, several "driver" genes are mutated at a high frequency in pancreatic cancer, and the altered physiology consequent to these mutations allows the tumor initiating clone to escape the regulatory controls ("niche"), leading to tumor formation (Jones et al. 2008; Yachida et al. 2010). Second, extensive histopathological analyses have led to the recognition of tangible noninvasive precursor lesions that exhibit, with variable frequency, the entire range of genomic alterations that characterize pancreatic cancer (*see Chapter by Offerhaus*) (Kanda et al. 2012; Maitra et al. 2003). Third, genetically engineered mouse models, in which one or more key-mutated genes are expressed in the pancreas, recapitulate the full spectrum of phenotypic alterations of the cognate human disease, from noninvasive precursor lesions (pancreatic intraepithelial neoplasia or PanINs) to metastatic pancreatic cancer (*see Chapter by Pasca di Magliano*) (Hingorani et al. 2003, 2005; Perez-Mancera et al. 2012). Fourth, an increased risk for developing pancreatic cancer has been shown in members of families affected by rare cancer predisposition syndromes (*see Chapter by Petersen*) (Jacobs et al. 2010; Canto et al. 2012). Affected individuals from such high-risk families often harbor germ line mutations that permit the emergence of pancreatic cancer over the lifetime of these patients (Couch et al. 2007; Jones et al. 2009).

The identification of genes involved in pancreatic cancer development was historically obtained through a candidate gene approach. With some notable exceptions (Hahn et al. 1996), the candidate approach was able to establish the role of frequently mutated genes or to identify critical pathways already described in other tumor types, but is inadequate in discovering unexpected molecular alterations or pathways. Recently, the advent of massively parallel high-throughput technologies, such as next-generation sequencing (NGS), has provided the possibility of interrogating cancer genomes at an unprecedented resolution (Wu et al. 2011a; Jiao et al. 2011; Stransky et al. 2011; Parsons et al. 2011; Bettegowda et al. 2011) (*and see chapter by Wei and Kumar*). The information provided by such sensitive methods is expected not only to increase our knowledge of the genetic landscape of human cancers but also, more importantly, to usher in an era of personalized medicine based on tumor-specific genetic aberrations. In the context of pancreatic cancer, there is considerable hope that the translation of new molecular targets into the clinical setting is likely to improve risk assessment, early diagnosis, and the identification of the best possible treatment for each individual patient. In this chapter we describe the spectrum of the most common genetic alterations ("mountains") that

drive the development of sporadic pancreatic ductal adenocarcinomas as well as less frequent alterations ("hills") (Vogelstein and Kinzler 2004a). Furthermore, new insights provided by novel high-throughput technologies and their translational relevance are also discussed.

The Genomic Landscape of Pancreatic Cancer: An Overview

Chromosomal Aberrations

Genomic instability represents a hallmark of pancreatic cancer, as well as other cancer types (Campbell et al. 2010; Stephens et al. 2011). Numerous alterations at the chromosomal level are seen in pancreatic cancer and, depending upon the underlying genetic mechanism, they can either occur as chromosomal instability (CIN) or microsatellite instability (MIN). This distinction, which appears to be mutually exclusive, is justified by the unique molecular and histological features of each type of alteration (Goggins et al. 1998; Wilentz et al. 2000).

CIN, which is revealed in the vast majority of pancreatic cancers (97 %) by cytogenetic analysis, is expressed through copy-number gains and losses, translocations, inversions, amplifications and homozygous deletions. Although such alterations may appear to be randomly distributed, they reflect a distinctive pattern in which selected genes that play a critical role in carcinogenesis are targeted and disrupted. In fact, a recent study has elucidated the concept of STOP (suppressors of tumorigenesis and proliferation) and GO (growth enhancers and oncogenes) that contribute negatively and positively towards the neoplastic phenotype, respectively (Solimini et al. 2012). In many instances, areas of hemizygous deletions are enriched for "islands" of high-density STOP genes that each contribute, on the basis of their haploinsufficiency, towards the eventual malignant phenotype, even in the absence of mutations on the remaining allele. Most frequently, numerical changes of the chromosomal architecture in pancreatic cancer are characterized by losses, particularly on chromosomes 6p, 9p, 13q, 17p, and 18q, as well as gains on chromosomes 7q and 20 (Mahlamaki et al. 2004; Holzmann et al. 2004). Several techniques have been used to identify regions of copy number alterations at a high resolution, including dense allelotyping and microarray analysis on single nucleotide polymorphism (SNP), bacterial artificial chromosome (BAC), oligonucleotide, or cDNA arrays (Calhoun et al. 2006; Nowak et al. 2005; Gysin et al. 2005; Chen et al. 2008; Bashyam et al. 2005; Shain et al. 2012; Kwei et al. 2008a). For example, Iacobuzio-Donahue et al. investigated chromosomal alterations in 80 pancreatic cancer xenografts by genome wide allelotyping, and confirmed losses in chromosomes 9p, 18p and 17p as the most common copy number alterations, with the regions of overlap encompassing three well known tumor suppressor genes in pancreatic cancer (*CDKN2A, SMAD4/DPC4 and TP53*, respectively) (Iacobuzio-Donahue et al. 2004). Of note, allelotyping of PanINs has revealed imbalances in several chromosomal regions also altered in pancreatic cancer, suggesting that CIN occurs early during

the progression from noninvasive precursor lesions to invasive adenocarcinoma (Luttges et al. 2001; Yamano et al. 2000). Kern and colleagues have identified two patterns of CIN in pancreatic cancer using high-density SNP arrays, "original" CIN, characterized by an admixture of allelic loss and copy number changes, and "holey" CIN, exemplified by large regions of homozygous deletions ("holes") in the genome (Calhoun et al. 2006).

The use of array-based approaches to study copy number alterations in pancreatic cancer have helped define the regions of amplification and deletion with unprecedented resolution, including at the level of individual or neighboring genes. Notably, there are many instances wherein genes or pathways are altered predominantly by copy number changes rather than mutations at the nucleotide level. For example, *MYC,* the gene encoding the master transcriptional factor C-myc and located on chromosome 8q, is amplified in 10–20 % of pancreatic adenocarcinomas (Nowak et al. 2005; Bashyam et al. 2005), although somatic mutations have not been reported in this cancer type. Transcriptional overexpression is also observed in the majority of cases (Han et al. 2002), further highlighting the importance of altered C-myc signaling in pancreatic cancer. As recent studies have shown, C-myc plays a crucial role in metabolic reprogramming of cancer cells, allowing them to thrive in the hypoxic, nutrient-deprived environs of the tumor microenvironment (Dang 2010, 2012). Another example of a region of recurrent amplification occurs on chromosome 18q, which targets the gene encoding the transcription factor *GATA6*, amplified in approximately a fifth of pancreatic cancers (Fu et al. 2008; Kwei et al. 2008b). As with *MYC*, somatic mutations of the GATA transcription factor family are rare in pancreatic cancer (Jones et al. 2008). Similarly, inactivation of genes whose encoded products are involved in chromatin remodeling (*ARID1A, ARID1B*, *PBRM1, SMARCA2*, and *SMARCA4*) can be seen in up to a third of pancreatic cancers, only a minor fraction of which occurs via somatic mutations and the majority through copy number alterations (Shain et al. 2012).

Telomere Alterations

Telomeres are tandem repeats of specific noncoding nucleotide sequences (TTAAGGG) present at the ends of chromosomes (Blackburn et al. 2006). Telomeres play a fundamental role as guardians of genomic integrity, protecting chromosomal ends from breakage or fusion with neighboring chromosomes. Since cell cycle results in progressive telomere shortening, telomere length can be maintained by activation of the enzyme telomerase, a feature observed in most human cancers (Harley et al. 1990; Martinez and Blasco 2011). Reactivation of telomerase protects cancer cells from critical telomere shortening and resulting DNA damage, thus allowing limitless replication. Telomerase activation is observed fairly late in the multistep progression of pancreatic cancer, however, and is preceded by an abnormal shortening of telomeres that occurs at the stage of noninvasive precursor lesions (van Heek et al. 2002a). Indeed, more than 90 % of low-grade PanIN lesions

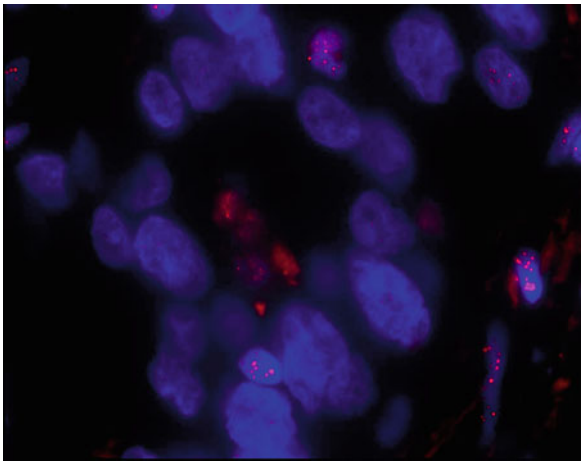

Fig. 1 Attrition in telomere length is one of the earliest detectable molecular alterations in pancreatic cancer, nearly ubiquitously observed at the stage of even low-grade PanIN lesions. A specific fluorescence in situ hybridization probe against telomeric DNA is used for semiquantitative measurement of telomere lengths in archival tissues (TEL-FISH). In this figure, a neoplastic gland from a ductal adenocarcinoma demonstrates near total loss of fluorescence intensity by TEL-FISH. In contrast, bright telomere signals are observed in the adjacent stromal cells, and one infiltrating lymphocyte at the bottom of the gland. Photomicrograph courtesy of Alan Meeker, PhD, Department of Pathology, Johns Hopkins University School of Medicine

demonstrate marked shortening of telomeres, as compared with normal pancreatic ductal epithelium, suggesting that telomere attrition is probably one of the earliest genetic events during pancreatic carcinogenesis (Fig. 1). While the basis for the near uniform telomere dysfunction in precursor lesions is unclear, it is likely that such dysfunction sets the stage for subsequent "breakage-fusion-breakage" cycles, which lead to chromosomal instability and frank neoplasia.

Oncogenes

Somatic activating mutations in the *KRAS2* gene are present in over 90 % of pancreatic adenocarcinomas and PanIN lesions, rendering it the most frequently mutated oncogene in this tumor type (Jones et al. 2008; Kanda et al. 2012). *KRAS2* gene (also known as Kirsten rat sarcoma viral oncogene homolog), located on chromosome 12p, encodes a GTP-binding and hydrolyzing enzyme involved in growth factor signaling pathways (Vigil et al. 2010). The K-ras protein activates multiple downstream effector pathways required for oncogenesis, including cell survival, cell proliferation, cell invasion, and aberrant cellular metabolism (*see chapter by Bar-Sagi*). Principal effectors of K-ras include the mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/Akt, and Ral signaling pathways,

among others (Young et al. 2009). Under physiological conditions, K-ras is transiently activated by GTP binding, followed rapidly by inactivation due to its intrinsic property of GTP hydrolyzation ("GTPase"). This endogenous GTPase activity is compromised by somatic mutations occurring in the GTP-binding pocket, which causes K-ras to remain constitutively active (DeNicola and Tuveson 2009; Perez-Mancera and Tuveson 2006). Interestingly, the vast majority of *KRAS2* point mutations in human pancreatic cancer are confined to codon 12, and less frequently to codons 13 and 61. In addition to invasive cancer, *KRAS2* mutations are also found in PanINs, including nearly all low-grade PanINs. As recently shown (Kanda et al. 2012), lower grade PanINs represent an admixture of mutant and nonmutant clones of cells, with a progressive increase in the proportion of the mutant clone accompanying histological progression to invasive neoplasia.

Recently developed animals models provide some of the most compelling evidence that K-ras is required for the initiation, maintenance, and progression of pancreatic cancer. Specifically, the expression of mutant *Kras* in the mouse pancreas during development is sufficient to yield the development of murine PanINs (mPanINs), which culminates in invasive adenocarcinoma in a fraction of animals (Hingorani et al. 2003; Aguirre et al. 2003a). More recent studies in transgenic animals have also underscored the importance of *Kras* in the maintenance of pancreatic cancer. This has been accomplished by the use of doxycycline-modulated *Kras* expression in the murine expression, wherein "turning off" mutant protein expression results in regression of established mPanINs and even invasive adenocarcinomas (Collins et al. 2012; Ying et al. 2012). Finally, mouse models of cooperation between mutant *Kras* and *p16* loss have found an intriguing loss of heterozygosity (LOH) of the wild-type *Kras* allele in advanced lesions (metastases), suggesting that the wild-type protein might interfere with the oncogenic function of the mutant K-ras protein (Qiu et al. 2011). In light of the near ubiquitous nature of *KRAS* mutations in pancreatic cancer, and the observed dependence in animal models on sustained Ras signaling, one presumes that pharmacological inhibition of mutant K-ras protein would be a therapy of choice in this malignancy. Unfortunately, clinical trials with inhibitors of farnesyltransferase, a key enzyme in the post-translational processing and membrane targeting of Ras protein, have been disappointing in pancreatic cancer (Kelland 2003; Van Cutsem et al. 2004). Several alternative strategies are currently undergoing evaluation, including targeting of Ras effectors pathways, either singly, or more increasingly, in combination (Feldmann et al. 2011; Collisson et al. 2011).

KRAS2 mutations also represent candidate biomarkers for the diagnosis of pancreatic cancer in biological samples such pancreatic juice, stool, and blood (Goggins 2005). However, in heterogeneous biological samples, the overwhelming presence of wild-type DNA, as opposed to a limited number of mutant molecules, renders *KRAS2* mutations particularly difficult to detect using conventional assays. To overcome these limitations, ultrasensitive assays for the detection of mutant *KRAS2* have been generated in the last few years, which are able to identify low-concentration mutant molecules and estimate differences in the proportion of mutant *KRAS2* molecules between pancreatic cancer and noncancerous conditions.

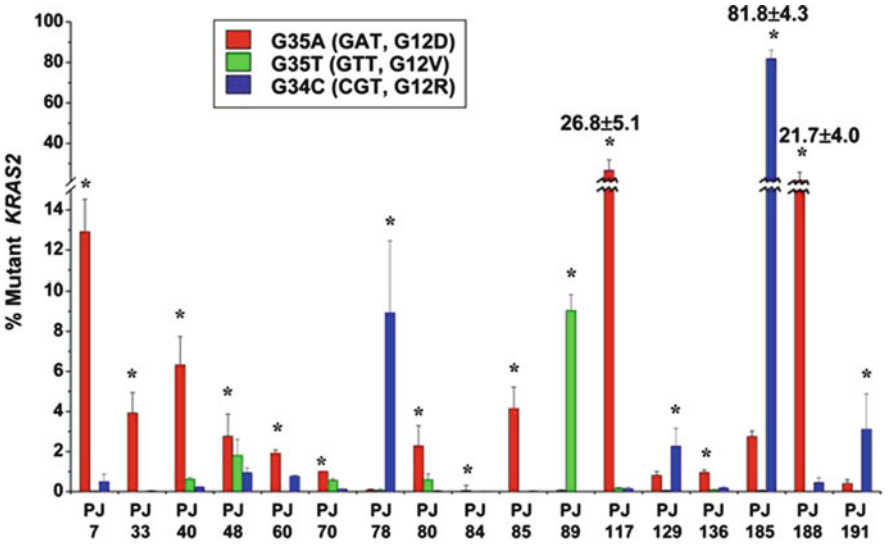

Fig. 2 Quantitative detection of mutant KRAS molecules in pancreatic juice samples obtained from patients with pancreatic adenocarcinoma using ultrasensitive LigAmp technology. Figure reproduced with permission from Shi C et al., Cancer Biol Ther 2008, Landes Bioscience Publishers, Austin TX

For example, a technique known as "LigAmp," which involves sequential DNA ligation and PCR amplification, has been recently developed to detect and quantify *KRAS2* mutant molecules in pancreatic juice samples (Shi et al. 2008) (Fig. 2). In another ultrasensitive approach, known as BEAMing (beads, emulsion, amplification, and magnetics), a single DNA molecule is assigned to a single magnetic bead, PCR-amplified and coupled with specific fluorescent-labeled oligonucleotides (Dressman et al. 2003; Diehl et al. 2008). The percentage of mutant DNA molecules in a mixed population of DNA molecules is then quantified by analyzing fluorescence emission through a flow cytometer. If validated by additional studies, it is expected that these new quantitative assays will greatly improve the diagnostic armamentarium available for the early diagnosis of pancreatic cancer.

In addition to the overwhelming dominance of mutant *KRAS2*, other pathway components can occasionally be altered, and might either be additive, or less frequently substitute for, mutant K-ras function. For example, in rare instances (~1 %), pancreatic cancers may harbor somatic *BRAF* mutations, and some studies have suggested that this preferentially occurs in the setting of *KRAS2*-wild type tumors (Calhoun et al. 2003). In this instance, one envisions that mutant *BRAF* gene product is driving activation of the MAPK signaling pathway. Similarly, amplification of the *AKT2* gene locus on chromosome 19q is observed in ~10 % of pancreatic cancers (Cheng et al. 1996; Ruggeri et al. 1998), and is typically co-existent with a mutant *KRAS2*, likely contributing the abnormal activation of signaling in the Akt oncogenic pathway.

Tumor-Suppressor Genes

The *CDKN2A/p16* gene on chromosome 9p21 is inactivated in more than 95 % of pancreatic cancers, representing the most frequently inactivated tumor suppressor gene in this tumor type (Maitra and Hruban 2008; Rozenblum et al. 1997; Caldas et al. 1994; Schutte et al. 1997). Unlike *KRAS* mutations, *CDKN2A/p16* inactivation occurs through multiple mechanisms: it is estimated that 40 % of the cancers harbor a homozygous deletion of both alleles of the gene, and another 40% presents an intragenic mutation in one allele coupled with loss of heterozygosity (LOH) of the second, reflecting classical Knudsonian mechanisms of gene inactivation (Knudson 1996). In the remaining 10–15 % of cancers, *CDKN2A/p16* gene is inactivated via promoter hypermethylation. Notably, abnormal p16 protein expression is also observed in 30 % of PanIN-1, 55 % of PanIN-2 and 70 % of PanIN-3, and similar to invasive neoplasia, the underlying genetic abnormalities occurs via a combination of gene mutation, promoter methylation, and allelic deletions (Moskaluk et al. 1997; Hustinx et al. 2005a; Fukushima et al. 2002). Germ line *CDKN2A/p16* mutations occur in the familial atypical multiple mole and melanoma (FAMMM) syndrome (*see chapter by Petersen*) (Fusaro and Lynch 2000). Persons affected by this syndrome characteristically present with numerous nevi, including dysplastic nevi characterized by atypical shape, size, and color and a predisposition for developing malignant melanoma. Notably, these patients also harbor nearly a 20-fold lifetime risk of developing pancreatic cancer (Klein et al. 2001), underscoring the importance of *CDKN2A/p16* as a tumor suppressor gene in this cancer type. The gene product of *CDKN2A/p16* regulates cell cycle progression by inhibiting cyclin D1-CDK4/6, a kinase complex that is involved in promoting the G1/S phase transition by inactivating the retinoblastoma protein, Rb (Sherr 2004). The *CDKN2A/p16* locus at chromosome 9p21 has an overlapping reading frame with *Arf*, whose gene product is involved in stabilizing p53 (Kim and Sharpless 2006). In genetically engineered mice, co-deletion of *Cdkn2a/p16* in conjunction with *Arf* plus expression of a mutant *Kras* allele in the pancreas results in rapidly progressive and lethal adenocarcinomas (Aguirre et al. 2003b). Subsequent studies have confirmed that pancreas-specific bi-allelic deletion of *Cdkn2a/p16* alone (with intact *Arf*) in association with mutant *Kras* is sufficient in generating murine pancreatic adenocarcinomas (Bardeesy et al. 2006).

The high frequency of *CDKN2A/p16* abnormalities (especially mutations and promoter methylation) renders this gene as an attractive candidate for biomarker studies. Not surprisingly, both classes of abnormalities of *CDKN2A/p16* can be identified in the pancreatic juice of patients harboring pancreatic cancer, especially using sensitive detection technologies (Bian et al. 2006; Matsubayashi et al. 2006). Interestingly, the gene encoding methylthioadenosine phosphorylase (MTAP), which resides approximately 100 kb telomeric to the *CDKNA2A/p16* gene, is frequently included in the 9p21 homozygous deletions, present in up to 1/3rd of pancreatic cancers overall (Hustinx et al. 2005b). The MTAP enzyme is critical for purine biosynthesis through the salvage pathway, and therefore, pancreatic cancers harboring

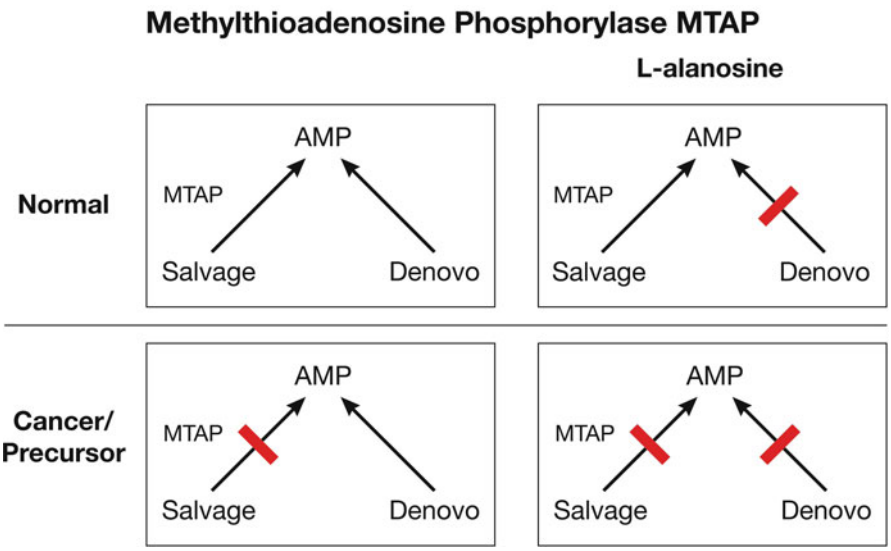

Fig. 3 Purine biosynthesis in cells occurs via either the de novo or the salvage pathways. Methylthioadenosine phosphorylase (MTAP) is the essential enzyme for purine synthesis through the salvage pathway. In pancreatic cancers with homozygous *MTAP* gene deletions, the tumor cells are dependent on de novo purine synthesis. In these cases, blockade with a systemic inhibitor of de novo synthesis like L-alanosine can provide a synthetic lethal effect that is restricted to cancer cells only

MTAP homozygous deletions are potentially susceptible to small molecule inhibitors of de novo purine biosynthesis, providing a great example of a synthetic lethal interaction that is targeted at a passenger, and not a driver alteration (Hustinx et al. 2005b; Karikari et al. 2005; Bertino et al. 2011) (Fig. 3).

The *TP53* gene, located on chromosome 17p, plays a critical role as a "guardian" of the genome. It regulates the G1/S cell cycle phase checkpoint, and induces cell cycle arrest in the setting of DNA damage; the inability to repair damaged DNA then triggers p53-dependent apoptosis (Vazquez et al. 2008). Somatic mutations of *TP53* gene are found in ~50–75 % of invasive pancreatic cancers, which results in the inability of the mutant protein to bind to DNA and activate the p53 transcriptional network (Jones et al. 2008; Hingorani et al. 2005). Several recurrent *TP53* mutations observed in human cancers, such as the R175H mutation, have a dominant-negative "gain-of-function" effect, which attenuates the function of the wild type allele (Jackson et al. 2005; Olive et al. 2004). Thus, loss of the second allele, although generally observed as a chromosome 17p loss of heterozygosity, may not always be necessary to abrogate physiologic p53 protein function. The majority of *TP53* mutations result in stabilization of the encoded protein, and this can be detected as nuclear accumulation of p53 on immunohistochemistry (Baas et al. 1994). In PanINs, nuclear p53 accumulation is typically detected at the stage of PanIN-3 and beyond, suggesting that it is a late anomaly in the multistep progression

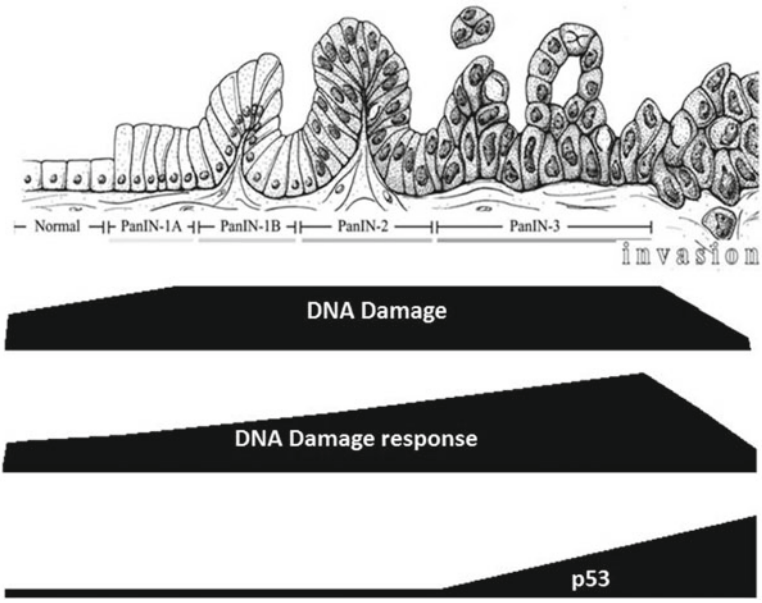

Fig. 4 Retention of p53 function acts as a crucial barrier to cancer progression in the pancreatic epithelium, in response to progressive accumulation of DNA damage and activation of the DNA damage response (DDR). Inactivation of p53 function at the stage of PanIN-3 and beyond is associated with bypass of the DDR checkpoint, and progression to invasive cancer. Figure reproduced with permission from Koorstra et al., Mod Pathol 2009

of pancreatic cancer (Maitra et al. 2003). This is in contrast to markers of DNA damage response (such as phosphorylated ATM and Chk2 proteins), which are observed even in the lowest-grade PanIN lesions (Koorstra et al. 2009). The retention of p53 function in low-grade PanINs (and the resulting checkpoint phenomenon) might explain why pancreatic cancers remain relatively uncommon despite the widespread prevalence of lower grade PanINs in the general population (>50 % harbor such noninvasive lesions above the age of 60 years) (Cubilla and Fitzgerald 1976). Loss of p53 function at the PanIN-3 stage "opens the floodgates" for progression to invasive neoplasia (Fig. 4). The high frequency of *TP53* mutations in pancreatic cancer provides an opportunity for its use as a biomarker in clinical samples, such as pancreatic juice samples (Bian et al. 2006). In addition, the recent development of mutant allele specific p53 targeted small molecule therapeutics (in particular, those that can reactive wild-type function in the R175H allele, the most common mutation in pancreatic cancer) (Yu et al. 2012), provides new therapeutic opportunities against the mutant protein. Another example of selective toxicity against p53-mutant pancreatic cancers has recently been identified in preclinical studies that targeted the Wee1 kinase, which inhibits Cdc2, using a potent and selective small molecule antagonist (Rajeshkumar et al. 2011). Specifically, agents that block Wee1 kinase function, and hence promote Cdc2-mediated G2-M progression result

in a phenomenon of so-called "mitotic catastrophe" in the setting of exacerbated DNA damage, such as that induced by concomitant therapy with antineoplastic agents like gemcitabine.

The *DPC4/SMAD4* gene, located on chromosome 18q, encodes for an intracellular protein that transduces growth inhibitory signals upon binding of transforming growth factor β (TGFβ) to its membrane receptors (Siegel and Massague 2003). *DPC4/SMAD4* functions as a key tumor suppressor gene, and homozygous deletion or intragenic inactivating mutation of *DPC4/SMAD4* occur in approximately 55 % of pancreatic adenocarcinomas (Hahn et al. 1996). Of note, loss of *DPC4/SMAD4* is infrequently to rarely seen in other pancreatic neoplasms, such as pancreatic neuroendocrine tumors (PanNETs), or in most extra-pancreatic epithelial neoplasms (Jiao et al. 2011; Schutte et al. 1996). This renders loss of Dpc4/Smad4 protein expression in metastases from occult primaries as a relatively specific, albeit not particularly sensitive, biomarker for pancreatic adenocarcinoma (Tascilar et al. 2001a; van Heek et al. 2002b). Mutations of *DPC4/SMAD4* gene in adenocarcinomas is the only one of the "big four" that has been shown to significantly correlate with decreased survival at both the genetic and protein level (the latter using immunohistochemistry in archival samples) (Blackford et al. 2009; Tascilar et al. 2001b). In addition, mutations of *DPC4/SMAD4* correlate with extensive systemic metastases in terminal pancreatic cancer patients, versus oligo-metastatic or locally advanced disease in those with retained function (*see chapter by Iacobuzio-Donahue*) (Iacobuzio-Donahue et al. 2009). In the multistep progression model, loss of Dpc4/Smad4 protein expression is observed as a relatively "late" event, mostly at the stage of high-grade PanIN lesions (Maitra et al. 2003). Recent chemical genetic approaches have identified compounds that are synthetic lethal to cells with DPC4/SMAD4 mutations, providing an opportunity for molecularly targeted therapies (Wang et al. 2006).

Other tumor suppressor genes have been shown to be inactivated at low frequency in pancreatic cancer (<5 %). Somatic mutations of the *LKB1/STK11* gene, which encodes for a serine threonine kinase, are rarely observed in sporadic pancreatic cancer, but more commonly in the setting of familial pancreatic cancer arising in patients with Peutz-Jeghers syndrome (Su et al. 1999). Individuals affected by this autosomal-dominant syndrome harbor an increased risk of developing colorectal hamartomatous polyps, as well as pancreatic cancer (Giardiello et al. 1987). The *LKB1* gene product is a multifunctional protein involved in metabolic sensing, maintenance of epithelial polarity and in regulating cytoskeletal architecture, amongst others (Hezel and Bardeesy 2008) (Fig. 5). In murine models, intraductal papillary mucinous neoplasms (IPMN) cystic neoplasms develop in the pancreas upon conditional *Lkb1* deletion (Hezel et al. 2008). Notably, loss of Lkb1 protein expression is observed in up to a third of cystic IPMNs of the pancreas (*see chapter by Offerhaus*) (Sahin et al. 2003), although somatic *LKB1* mutations were not seen in the recent sequencing of the IPMN exome (Wu et al. 2011a). Intragenic mutations and homozygous deletions of the *MKK4* gene occur in <5 % of pancreatic cancers (Su et al. 1998). The *MKK4* gene, located on chromosome 17p, encodes for a component of stress-activated protein kinase cascade and plays a role in growth

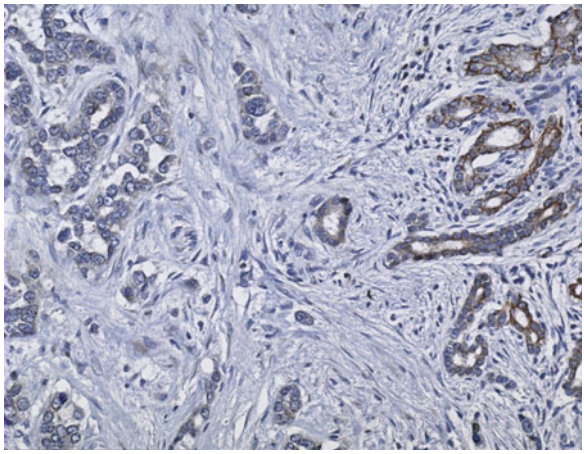

Fig. 5 Loss of Lkb1/Stk11 protein expression by immunohistochemistry in a pancreatic ductal adenocarcinoma. The neoplastic glands (*left half*) are negative for Lkb1 expression, while the intermixed normal ductal epithelium (*right half*) demonstrates robust labelling

control and apoptosis (Robinson et al. 2003; Haeusgen et al. 2011). Furthermore, inactivation of the *MKK4* gene has been documented in subsets of metastatic pancreatic cancer lesions, suggesting that the product of this gene may act as a metastasis suppressor (Xin et al. 2004).

Genome-Maintenance Genes

In addition to oncogenes and tumor-suppressor genes, a third class of genes, collectively defined as genome-maintenance genes, is occasionally inactivated in pancreatic cancer (Vogelstein and Kinzler 2004b). Also known as "caretakers," these genes are involved in the repair of DNA breaks, minimizing errors during DNA replication. One of the most commonly inactivated "caretaker" genes, in approximately 5 % of sporadic pancreatic cancers, is the *BRCA2* gene, located on chromosome 13q (Jones et al. 2008; Naderi and Couch 2002). Germ line mutations of *BRCA2* are observed in 5–10 % of patients with an inherited predisposition to pancreatic cancer, and have a particular propensity to occur in families of Ashkenazi Jewish heritage (*see chapter by Petersen*) (Ozcelik et al. 1997; Goggins et al. 1996; Hahn et al. 2003; Lal et al. 2000). The product of *BRCA2* interacts with proteins encoded by the Fanconi anemia genes (the *FANC* genes) to mediate homologous recombination at sites of DNA double-strand breaks (Gudmundsdottir and Ashworth 2006). Notably, pancreatic cancers that harbor bi-allelic mutations of *BRCA2* are characterized by exquisite sensitivity to DNA cross-linking agents (e.g., mitomycin C, cisplatin) as well as poly (ADP-ribose) polymerase inhibitors (PARP-i), providing an avenue for "personalized" therapy in this malignancy (Gallmeier and Kern 2007; van der Heijden et al. 2005; James et al. 2009). Recently, mutations have also been described in other components of the Fanconi anemia pathway, such as the *Partner and Localizer of BRCA2* (*PALB2*) gene, which encodes for a partner that spatially

localizes BRCA1 and BRCA2 proteins at sites of double strand breaks, in order to facilitate repair (Jones et al. 2009). Pancreatic cancers with bi-allelic *PALB2* mutations are similarly sensitive to the effects of cisplatin and mitomycin C (Villarroel et al. 2011). One of the important caveats that have emerged from mouse models of conditional *Brca2* deficiency in the pancreas is that haploinsufficiency for *Brca2*-function might be sufficient for inducing exocrine neoplasia, particularly in combination with mutant *Kras* (Skoulidis et al. 2010). This has therapeutic implications for treating "*BRCA*"-associated human pancreatic adenocarcinomas with PARP-i, since retaining a functional *BRCA2* allele would potentially render the tumors resistant to this class of agents (Fong et al. 2010). The data on somatic loss of the second *BRCA2* allele in pancreatic adenocarcinomas arising in patients with a germ line defect of one allele remains controversial, with at least one study suggesting that it may be retained, rendering such tumors resistant to PARPi-based therapies (Skoulidis et al. 2010).

Other genes involved in DNA repair that have been implicated in pancreatic carcinogenesis include *hMLH1* and *hMSH2*, mostly in the context of familial pancreatic cancers arising on the backdrop of hereditary non-polyposis colorectal cancer (HNPCC) (Lindor et al. 2011; Ghimenti et al. 1999; Yamamoto et al. 2001). Mutations or transcriptional silencing in *hMLH1* and *hMSH2* have been shown to result in replication errors in simple repetitive units known as microsatellites (Parsons et al. 1993; Malkhosyan et al. 1996; Eshleman and Markowitz 1996). As a consequence, microsatellite instability (also known as a defect in mismatch repair or MMR) defines a unique genomic landscape, characterized by very few alterations in chromosome ploidy. Interestingly, pancreatic carcinomas with microsatellite instability exhibit a unique histological pattern, termed as "medullary," comprised of poorly differentiated histology, pushing borders, and large numbers of tumor infiltrating lymphocytes (Wilentz et al. 2000). As additional evidence of the distinct genetic basis for these neoplasms, mutations in the *KRAS2* gene are uncommonly seen in medullary carcinomas (Goggins et al. 1998).

New Perspectives from Exomic and Next-Generation Sequencing Studies

As previously stated, historically, the discovery of molecular alterations in human cancer was based on a candidate gene approach. These methods allowed researchers for the identification of frequently mutated genes (*KRAS*, *CDKN2A/p16*, *SMAD4/DPC4*, *TP53*) in pancreatic adenocarcinoma, although they were often unable to find genes altered at low frequency or in unexpected cancer pathways. The first comprehensive glimpse into the genomic landscape of pancreatic cancer came in 2008, with an exomic sequencing study performed on a series of 24 cancers (Jones et al. 2008). This study utilized automated Sanger sequencing for exome analysis, combined with serial analysis of gene expression (SAGE) for the transcriptome and genome-wide single nucleotide polymorphism (SNP) microrrays for copy number

aberrations, in order to generate an integrated assessment of molecular alterations in pancreatic cancer. Using this approach, the sequences of 23,219 transcripts, representing 20,661 protein-coding genes (99.6 % of the coding genome) were determined. Overall, 1,562 somatic mutations were identified, mostly represented by single base substitutions [missense and nonsense mutations, or insertions/deletions (i.e., "indels")]. Pancreatic cancers were found to harbor a median of 66 somatic mutations per tumor. Only a small proportion of the compendium of mutated genes within an individual sample actually contributes to tumorigenesis ("driver genes") and the vast majority simply represent a bystander effect of ongoing genetic instability and clonal evolution ("passenger genes") (Bozic et al. 2010). Genes with a minimum of two genetic alterations (at least one of which was predicted to result in altered function) and a mutation rate > 10 mutations/Mb, calculated by integrating gene size, nucleotide composition and other characteristics, were considered as candidate driver genes ("CAN" genes). Consequently, genes that did not fit these criteria were considered passenger genes. Such an approach led to the identification of 91 CAN genes. Of these, the previously known "big four" (*KRAS2, CDKN2A/p16, TP53, SMAD4/DPC4*) constituted the most obvious "mountains" on the genomic landscape. The rest of the landscape was comprised of low-frequency "hills" and even "private" (unique) mutations, underscoring the considerable genetic heterogeneity amongst the different tumor samples studied. These results might at first appear discouraging to researchers and clinicians in terms of developing targeted therapies. However, such a complexity is significantly reduced if altered genes are considered in the much broader context of biological pathways. In fact, 12 core biological pathways appear to be altered in most cases of pancreatic cancer, many of which are well-established hallmarks of cancer (Hanahan and Weinberg 2011) (Fig. 6). This information may harbor implications for the development of new therapeutic agents that target functional pathways or processes rather than individual products of mutated genes.

Although detailed discussion of the 12 core signaling pathways is beyond the scope of this chapter, one notable theme that has emerged from the pancreatic cancer exome sequencing effort (Jones et al. 2008), as well as other comparable solid tumor studies, has been the emergence of epigenetic modifiers as a major target of genomic alterations (Parsons et al. 2011; Jones et al. 2010, 2012; Varela et al. 2011; Fujimoto et al. 2012). Pancreatic cancers harbor widespread epigenetic alterations, which mimic the multistep genetic progression observed with coding sequences (*see chapter by Goggins*). It is postulated that many of the genomic alterations in chromatin modifying genes represent epigenetic "drivers" of cancer (Elsasser et al. 2011). For example, somatic mutations of the mixed-lineage leukemia 3 (*MLL3*) gene is observed in ~10 % of pancreatic cancers, rendering it as the fourth most commonly mutated tumor suppressor gene in this neoplasm (Jones et al. 2008). The protein encoded by *MLL3* encodes for a histone methyltransferase, which forms part of a multimeric complex involved in regulation of chromatin remodeling (Lee et al. 2009). As previously stated, numerous other chromatin modifying genes are inactivated by copy number alterations in pancreatic cancer (for example, *ARID1A*, *BRG1*, *PRBM1*), with almost a third of tumors demonstrating aberrations in this class of genes (Shain et al. 2012).

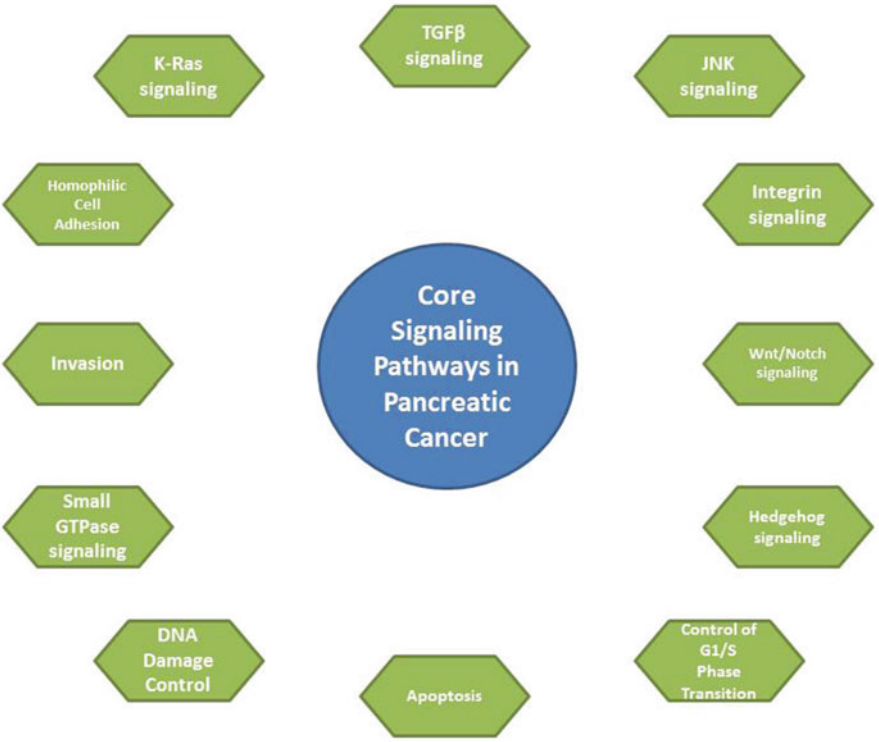

Fig. 6 Core signaling pathways that are altered by somatic mutations in the majority of pancreatic cancers. New data from the ICGC suggests that axonal guidance genes are another important category to be added to this list of core pathways. Figure adapted from Jones et al., Science 2008

The pancreatic adenocarcinoma exome has also been sequenced as part of an international effort known as the International Cancer Genome Consortium (ICGC) (Hudson et al. 2010). In contrast to the Jones et al. study (Jones et al. 2008), the pancreatic cancer ICGC team (led by investigators in Australia and Canada) utilized NGS technology on ~100 primary (Stage I and II) tumors (Biankin et al. 2012). Their data has reaffirmed many of the mutational "mountains" and "hills" uncovered in the Jones study, but also identified novel recurrent mutated pathways in pancreatic cancer. In particular, genes involved in embryonal axonal guidance [members of the *SLIT/ROBO* family of genes (Killeen and Sybingco 2008)] has emerged as recurrently mutated in pancreatic cancer, and appear to impart an adverse prognosis in patients bearing tumors with such somatic alterations.

The pancreas is one of the few organs where not only the most common neoplastic subtype (i.e., ductal adenocarcinoma) has been sequenced at the exome level, but so have nearly all other solid and cystic variant neoplasms as well (Wu et al. 2011a, b; Jiao et al. 2011). These studies, accomplished by harnessing the prowess of NGS have confirmed that "genetics begets morphology"—in that each of the histogenetic subtypes of pancreatic neoplasms is characterized by a unique underlying genomic

signature and driver gene mutations. For example, in contrast to ductal adenocarcinomas, PanNETs rarely, if ever, harbor mutations of the "big four" (*KRAS2, CDKN2A/p16, TP53, SMAD4/DPC4*) (Jiao et al. 2011). In contrast these lesions have three "mountains" on their genomic landscape—mutations of *MEN1*, germ line mutations of which are responsible for multiple endocrine neoplasia, type 1 (Marx et al. 1999); mutations of genes in the mammalian TOR signaling pathway (*PIK3CA*, *PTEN*, and *TSC2*) that determines susceptibility to inhibitors of TOR kinase (Meric-Bernstam et al. 2012; Yao et al. 2011); and a novel cancer pathway involving mutations of two genes—*DAXX* and *ATRX*, which encode for proteins that act as histone chaperones at telomeric DNA (Jiao et al. 2011). Mutations of *DAXX* or *ATRX* are found in a mutually exclusive manner in ~50 % of PanNETs, and result in a phenomenon called alternative lengthening of telomeres (ALT), characterized by absence of telomerase activity and abnormally long telomeres within neoplastic cells (Heaphy et al. 2011a). Of note, neither mutations of *ATRX/DAXX*, nor the ALT phenomenon have been described in ductal adenocarcinomas (Heaphy et al. 2011b). Similarly, the genomes of cystic mucinous neoplasms of the pancreas—including IPMNs and mucinous cystic neoplasms (MCNs) have recently been profiled, and approximately half contain inactivating mutations of *RNF43*, a gene encoding for RING domain containing ubiquitin ligase (Wu et al. 2011a). Mutations of *RNF43* have not been described in ductal adenocarcinoma, and the substrates of this ubiquitin ligase could represent the essential proteins responsible for driving exocrine neoplasia along a mucinous and cystic pathway. Recent studies suggest that RNF43 protein functions as a Wnt pathway inhibitor (Hao et al. 2012), and in conjunction with activating *CTNNB1* mutations in a subset of IPMNs (Chetty et al. 2006), aberrant Wnt activation might represent one of the mechanisms by which unique histogenetic differentiation occurs in cystic neoplasms versus "usual" ductal adenocarcinomas.

Conclusion

In conclusion, tremendous advances have been achieved over the last few years in our knowledge of the genomic alterations in spoardic pancreatic cancer. The application of NGS technologies has greatly expanded the scenarios in pancreatic cancer wherein this knowledge can be applied, from developing ultrasensitive early detection assays in biological specimens to more efficacious personalized therapies. In addition, knowledge gleaned from sequencing of the sporadic pancreatic cancer genome has been useful in expanding to the study of genomic alterations in precursor lesions (*see chapter by Offerhaus*) (Wu et al. 2011a, b), discovery of genes involved in familial pancreatic cancer (*see chapter by Petersen*) (Jones et al. 2009; Roberts et al. 2012), to elucidate the genomic complexity of metastases, and construct a timeline for progression to terminal disseminated cancer (*see chapter by Iacobuzio-Donahue*) (Yachida et al. 2010; Campbell et al. 2010). The public dissemination of sequence data using online portals such as the "ICGCMart" (Zhang et al. 2011) is likely to impact research and drug discovery efforts in pancreatic cancer for the next decade.

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