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Unravelling Cancer Signaling Pathways: A Multidisciplinary Approach

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To all the departed souls who lost their battle against cancer; to all the bravehearts who emerged victorious; and to all the indomitable spirits who did not give up.

Preface

As scientists working in the field of cancer biology, we often come across an innocent yet heartrending question: *When will the drug to treat cancer be invented?* While understanding the concern of the common people and empathizing with the unending sufferings of the patients and their families, we accept the fact that things are not as simple as they appear to be. Although cancer research has significantly evolved in the past few decades, yet a panacea for a multiplex disease like cancer seems far-fetched.

The complexity of cancer is defined by its heterogeneity and the fact that it is not a single but a group of diseases. Cancer is manifested by the disruption of several cellular processes, including cell cycle, cell death or apoptosis and deregulation of signal transduction pathways. It is also a consequence of deleterious mutations in several important genes and aberrant post-translational modifications in proteins that play critical roles in maintaining cellular integrity. Some types of cancer are often referred to as *malignant tumours* that differ from the benign ones due to their ability to move to other parts of the body through a process called *metastasis*. Based on the characteristics of a malignant tumour, several hallmarks of cancer have been defined, which include self-sufficiency in growth signals, insensitivity to anti-growth signals, uncontrolled growth, evasion of apoptosis, uninterrupted angiogenesis, genomic instability, metabolic reprogramming, and tissue invasion. Furthermore, a plexus of normal cells surrounding the tumour develops a propensity toward the acquisition of hallmark traits by creating the *tumour microenvironment*.

With a humble effort to dissertate the intricacies of the molecular pathways leading to cancer progression and ways of disease intervention, this book has been envisaged. It has been divided into 20 chapters that provide a comprehensive compilation of basic understanding of the intricate cellular networks and their anomalous behaviour that leads to the development of different types of cancers. It also elaborates on tools encompassing computational research to in vivo models for delineating these pathways at the molecular level. Understanding the complexity of the cellular network and recognizing the overall applicability of these concepts will pave a way toward the development of new means to combat cancer.

We wholeheartedly thank all the authors and contributors for their thoughtful insights on the different aspects of cancer biology. We are also grateful to our students and lab members who spent a considerable amount of time proofreading and giving invaluable inputs.

Navi Mumbai, India — Kakoli Bose
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Dr. Kakoli Bose is currently working as Scientific Officer at the Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Mumbai, India, where she is the Principal Investigator of Integrated Biophysics and Structural Biology Lab. Recently she has published a contributed volume as sole Editor of the book with Springer in 2015 titled *Proteases in Apoptosis: Pathways, Protocols and Translational Advances* {ISBN: 978-3-319-19496-7 (Print); 978-3-319-19497-4 (Online)}. The book has obtained 627 combined book chapter downloads since August 2015.

She obtained her Ph.D. from North Carolina State University, Raleigh, NC, USA, in 2003, and carried out post-doctoral work at Tufts University School of Medicine, Boston, USA, until 2007 before joining ACTREC as a scientist. She has several publications in reputed international journals to her credit. Dr. Bose has a vast experience in cancer cell signalling, and being a biophysicist and cancer biologist by training, she brings forth a multidisciplinary and an innovative essence to the field of biomedical research. She is an Associate Editor of Bioscience Reports and an Editorial Board Member of Biochemical Journal, Portland Press, UK. She is also a member of several prestigious associations and societies, such as American Chemical Society, Biochemical Society, Protein Society and Indian Association for Cancer Research. She has received various fellowships and awards in her scientific career, including the National Women Bioscientist Award, Govt. of India.

Dr. Pradip Chaudhari is currently working as a Scientific Officer at Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Mumbai, India. He obtained his Ph.D. from the University of Mumbai, India, in 2008. He has also worked at the prestigious Bhabha Atomic Research Centre, Mumbai, before joining ACTREC. Dr. Chaudhari is widely known for his expertise in preclinical imaging in cancer and biological evaluation of diagnostic and therapeutic radiopharmaceuticals in India and also abroad. He is a visiting/adjunct faculty at several national institutes/universities and received various fellowships and awards in his scientific career, including BOYSCAST Fellowship from the Department of Science and Technology, Government of India. He has pioneered nuclear medicine diagnostic applications for veterinary oncology patients. This has led to a dedicated Veterinary Nuclear Medicine Centre at Bombay Veterinary College, Mumbai. He

has played an instrumental role in biological evaluation of various diagnostic and therapeutic radiotracers.

Dr. Chaudhari is an Ad hoc Specialist of AAALAC International, USA, and has also been appointed as an Expert Member of Laboratory Advisory Committee, National Cancer Institute – All India Institute of Medical Sciences (NCI-AIIMS), New Delhi, India. He has 29 research articles published to his credit and 3 book chapters. He is editorial board member and reviewer of many reputed international journals and also a member of several prestigious societies, such as Society of Nuclear Medicine, Indian Association for Cancer Research and so forth.

Cancer Biology and Its Treatment Modalities: A Brief Historical Perspective

1

Aasna L. Parui and Kakoli Bose

Abstract

For thousands of years, tremendous amount of research has been carried out to understand one of the leading causes of death – cancer. Being known for its complexity, researchers have put tremendous efforts in acquiring every bit of knowledge that is required to understand the distinct aspects of tumor biology, progression, invasion, and metastasis. These include understanding the tumor physiology, developing different detection techniques, as well as identifying the genetic roots of the disease. This in turn would help in unraveling the signaling circuitry that regulates intercommunication within the tumor microenvironment. The main objective of this chapter is to provide a comprehensive overview of the different historic events that have taken place in the field of cancer research. Besides, it also briefly describes the different hallmarks of cancer that have been put forth to elaborate the different mechanisms adapted by cancer cells for their survival and progression.

Keywords

Cancer · Tumor biology · Detection · Signaling · History · Hallmarks of cancer

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1.1 Introduction

Cells are most commonly defined as the *basic building blocks* or *fundamental units* of life. They are known to form discrete functional packages in an organized pattern to build up an entire organism. For the proper maintenance of homeostasis, cells are constantly involved in sending and receiving tremendous amount of information among each other through the means of chemical signaling molecules. This form of communication governs and coordinates all the basic activities of a cell – including cell growth and development, tissue repair, strengthening of immunity, removal of toxic components, as well as balancing the mediums of cell death pathways. Any form of variation in these signaling interactions may lead to erroneous interpretation of the cellular information processing, thus hampering the homeostasis and leading to different forms of diseased states.

Cancer is one such highly complex disease that arises due to dysregulation of various signal transduction networks, which overall govern the molecular communications and major cellular processes. Under normal circumstances, individual cells or group of cells undergo expansion in response to distinct regulatory signals that govern their ability to progress through different stages of cell cycle and perform their primary function within the provisional microenvironment. However, in case of cancer, specific genetic mutations affect the functioning of signaling molecules and in turn give rise to a loss of control over critical cellular functions. Any fault in the processing of a particular aspect of the multistep and well-connected signaling pathway may result in impairment of the entire signaling network and eventually lead a normal cell to acquire cancer phenotype.

Being one of the leading causes of deaths globally, significant advances have been made in understanding tumor biology, tissue invasion, and metastasis. With decades of research undertaken to comprehend distinct arenas of cancer biology – from understanding the tumor physiology to developing different detection techniques and from identifying the genetic roots to deciphering the signaling circuitry that controls the intercommunication between various cells within the tumors – research in this field has significantly evolved. This chapter elaborates the 4000-year history of cancer and how the story has unfolded over the period of time, giving us more in-depth insights into the realm of cancer biology and expanding the horizons of avenues in cancer treatment.

1.2 Historical Perspective

Cancer has been known to the human race for over a long period of time, starting way beyond the introduction of its Greek terminology “karkinos” by the well-known Greek physician Hippocrates in 460 BC. The oldest records for human bone and breast cancers have been found in the ancient Egyptian mummies dating back to 1600–1500 BC (Sudhakar 2009). In the past 250 years, there have been a huge number of researches undertaken to comprehend the core of cancer biology, the signaling pathways involved, and the advancements in the treatment regimens.

In 1775, Sir Percivall Pott, an English surgeon and one of the founders of orthopedics, successfully established the relationship between exposure to chimney soot and the incidence of squamous cell carcinoma of the scrotum among chimney sweepers (National Cancer Institute 2015). He was the first scientist to report the effect of environmental exposure on the development of cancer (Fig. 1.1). In 1863, German researcher Rudolf Virchow identified an increase in the number of leukocytes in the blood specimens of cancer patients. He coined the term "leukemia" to describe this condition and proposed a link between inflammation and cancer. Further, in 1886, a Brazilian ophthalmologist Hilário de Gouvêa studied a case of childhood retinoblastoma and provided the first-ever documented evidence explaining the link between cancer and its inheritance. In 1895, Wilhelm Conrad Röntgen discovered the X-rays, and subsequently, in 1896, Emil Grubbe experimented with the use of X-rays in the treatment of cancer. With the discovery of radioactivity by Marie and Pierre Curie in 1898, the use of radium in radiation therapy for cancer treatment also began in few years. In 1902, Theodor Boveri proposed that alterations at the chromosomal level in a single cell may lead to activation of cellular pathways promoting uncontrolled cell division that subsequently led to cancerous tumor formation. Further, Paul Ehrlich in 1909 put forth the "immune surveillance" hypothesis, which suggested that the immune system usually suppresses tumor formation and, thus, provided the first fundamental principle of chemotherapy.

The beginning of the nineteenth century involved identification of the etiologic agents responsible for causing cancer. In 1911, Dr. Peyton Rous, a young American pathologist, discovered that the supernatant from tumor cells of Plymouth Rock hen contained a transmissible virus that was an etiologic agent causing spindle-cell sarcoma. Although he was the pioneer in identifying that some cancers are caused by infectious agents, this discovery gained importance only in the late 1950s. Further, Temin and Rubin demonstrated that a number of additional viruses could induce transformation in tissue culture cells and tumors in appropriate animal models (Frank 2011). This discovery expanded research into oncogenic retroviruses, which were known for their ability to direct DNA synthesis from an RNA genome through a polymerase commonly termed as *reverse transcriptase*. Studies on the replication of these oncogenic viruses, mechanisms by which their viral oncogenes integrated into the host genome, and subsequent expression of these oncogenes that induced malignant transformation were gaining popularity in the scientific community. This fetched Rous the Nobel Prize in 1966 for identifying the mode of malignant transformation by a virus that was named "Rous sarcoma virus (RSV)" in his honor. Besides, George Papanicolaou developed the *Pap smear test* in 1928 for the detection of cervical cancer in a precancerous state (National Cancer Institute 2015). The subsequent clinical trial was launched in 1952 and was known as "the largest clinical trial of secondary prevention in the history of cancer," as it provided significant advancement in the detection of cancerous cells till today.

The era of the 1940s marked three decades of intense growth in the field of cancer treatment. In 1941, Charles Huggins discovered that the removal of testicles caused reduction of testosterone levels and the subsequent administration of estrogens promoted the regression of prostate tumors. Thus, hormonal therapy came into

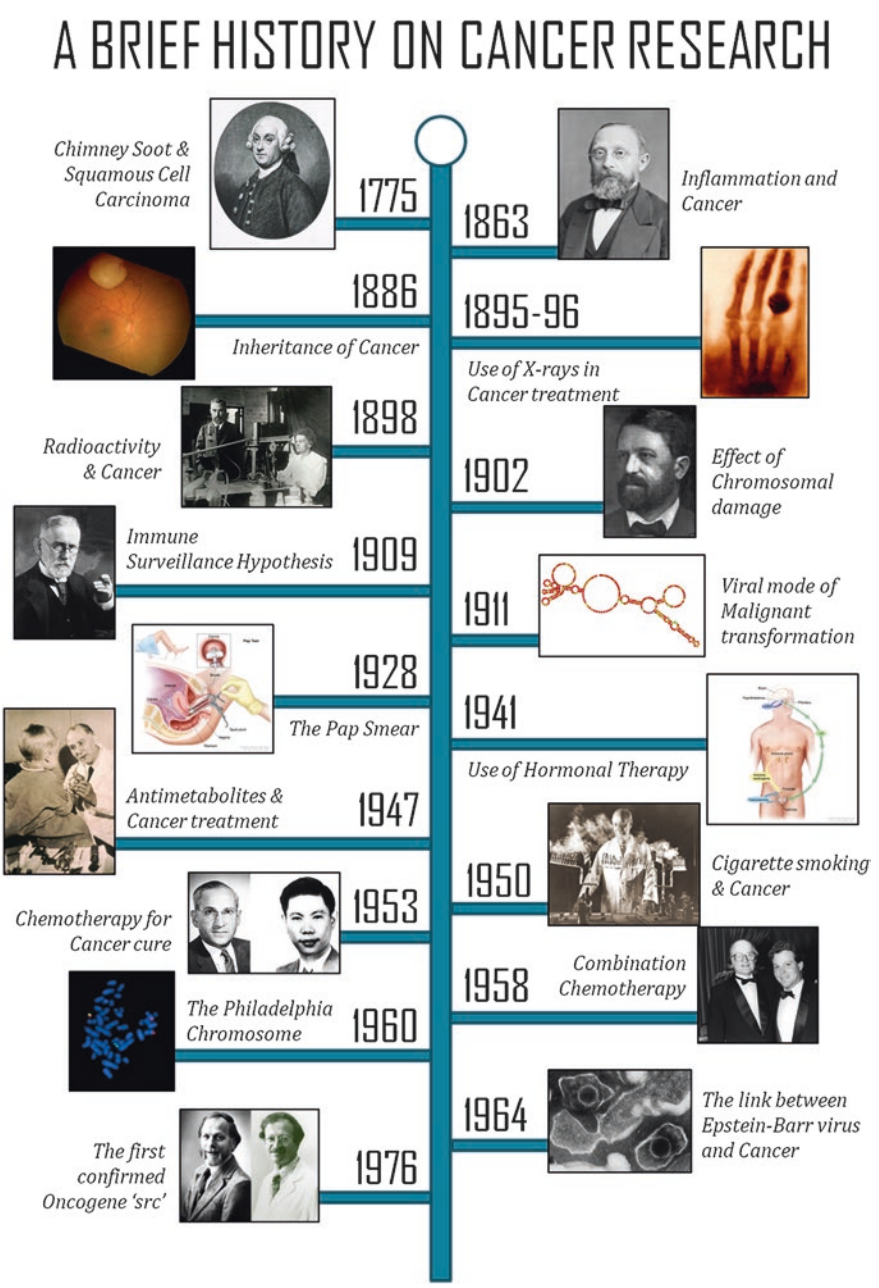

Fig. 1.1 A brief history of cancer research. This timeline illustrates the important events that have taken place in the field of cancer biology. (Images taken from (National Cancer Institute 2015) and Google images)

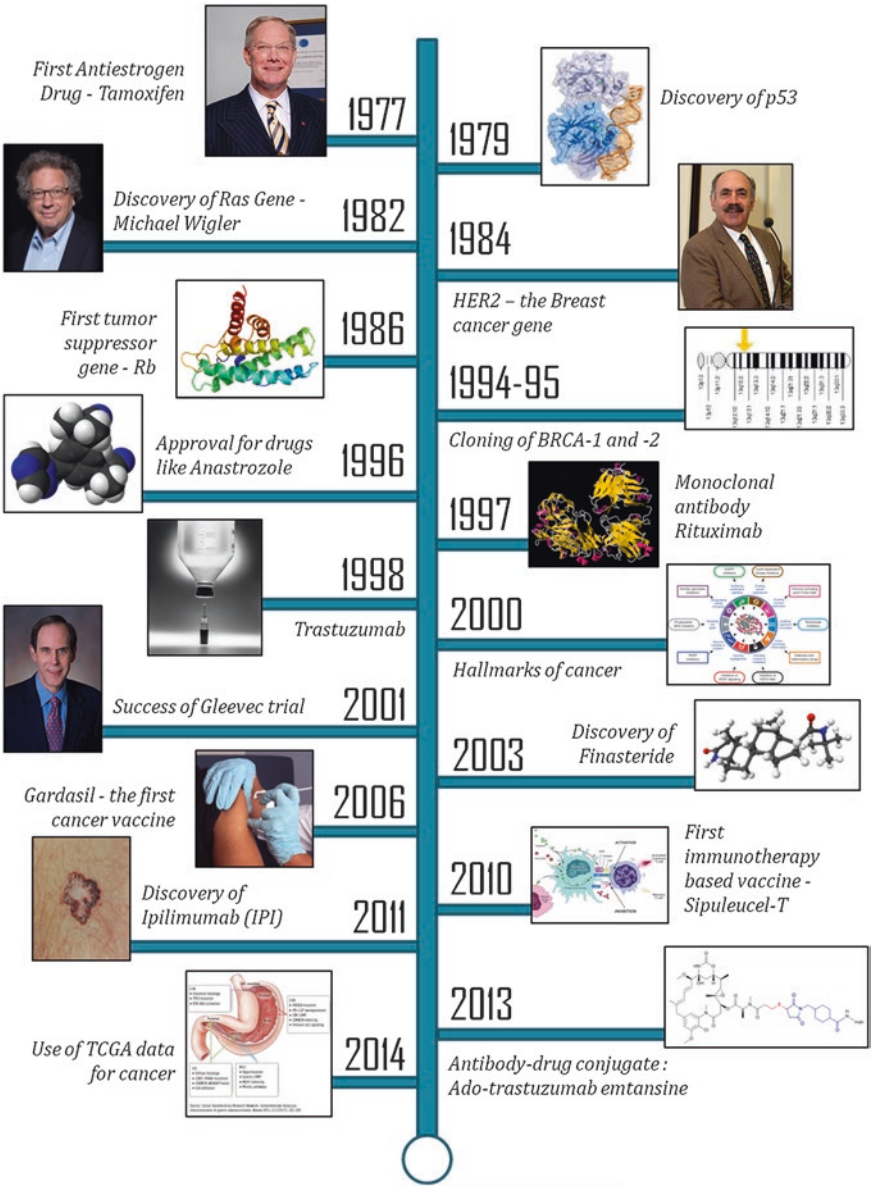

Fig. 1.1 (continued)

mainstream application for prostate cancer treatment. In 1947, Sidney Farber, a world-renowned pediatric pathologist, demonstrated that folic acid stimulated leukemic cell growth, while treatment with the antimetabolite drug aminopterin induced remissions of acute leukemia in children (Benner 2015). Although the antimetabolite drugs were known to be structurally similar to the biomolecules required for cellular processes such as DNA synthesis, they induce cell death by blocking these important processes. In 1950, Ernst Wynder, Evarts Graham, and Richard Doll identified cigarette smoking to be an important factor responsible for the development of lung cancer. With the use of the drug methotrexate for chemotherapy treatment of a patient with choriocarcinoma (a rare cancer of the reproductive tissue that mainly affects females), Roy Hertz and Min Chiu Li achieved the first complete cure of a human solid tumor in 1953. Subsequently in 1958, it was shown that combination chemotherapy with the drugs 6-mercaptopurine and methotrexate not only could induce partial as well as complete remissions but also prolonged the survival rates in patients with acute leukemia. Developed by Emil Frei and Emil Freireich, the combination therapy became more famous through the VAMP protocol trial (vincristine, amethopterin, mercaptopurine, prednisone) that was used to identify the different mechanisms by which each drug provides better treatment for cancer. In the case of chronic myelogenous leukemia (CML), Peter Nowell and David Hungerford in 1960 identified an unusually small chromosome (known as the Philadelphia chromosome) in the cancer cells of 95% patients. In 1964, the Epstein-Barr virus (EBV) was identified for the first time to be the etiologic agent for a number of human cancers, including nasopharyngeal carcinoma, Hodgkin lymphoma, and some gastric (stomach) cancers.

In 1969, the term "oncogene" was coined from the Greek word "onkos" meaning mass or load, and it describes the potential of a particular gene to cause cancer. Consequently, the first confirmed cellular oncogene "src" was discovered in 1976 by Dominique Stehelin, Harold Varmus, J. Michael Bishop, and Peter Vogt. Using an oncogenic retrovirus, they demonstrated that the DNA of normal chicken cells contained a gene related to the oncogene of avian sarcoma virus. The oncogene in the virus did not represent a true viral gene but was a normal cellular gene, which the virus had acquired during replication in the host cell and thereafter carried along. In this way, they identified the growth-controlling oncogenes in normal cells, and this finding led Bishop and Varmus to win the Nobel Prize in 1989. Another commendable discovery showed that the src-encoded protein displays an intrinsic protein tyrosine kinase (PTK) activity (Hunter and Sefton 1980).

Besides the discovery of viral mode of transformation, the start of the 1970s provided three competing theories about carcinogenesis. They were based on causes that resulted in transformation of normal cells into cancerous forms and were identified as viral, environmental, and biological (malfunctioning genes). Although none of the theories could alone stand to present the full story of cancer, each of these theories could eventually be proven correct in individual cases. In 1971, Dr. Judah Folkman proposed that angiogenesis plays a critical role in cancer development and that preventing this process can inhibit tumor growth by starving the tumor of vital nutrients (Emory University). In 1977, the US Food and Drug Administration (FDA)

approved the first antiestrogen drug – tamoxifen. The drug belonged to a class of selective estrogen receptor modulators, or SERMs, and was approved for breast cancer therapy. The year 1979 marks the landmark discovery of the most widely studied and commonly mutated gene in human cancer, the TP53 gene (also called p53). The protein product (p53 protein) was found to be a tumor suppressor, as it controlled cell proliferation and suppressed tumor growth. Further, in 1982, three different labs (Mariano Barbacid and Stuart Aaronson at the NIH, Robert Weinberg at MIT, and Michael Wigler at Cold Spring Harbor Laboratory) isolated the same gene called *RAS* from cancer cells. This viral gene encodes for 21 kDa (p21) proteins that belong to a class of small GTPases and have been found to be involved in cellular signal transduction. Thus, over-activation or expression of mutationally active form of the Ras protein was found to cause a number of cancers. In 1984, a new oncogene named "neu" was discovered in rats, and the human counterpart of this gene, HER2, was found to be overexpressed in the more aggressive form (~20%–25%) of breast cancers, known as HER2-positive breast cancers. Subsequently, in the year 1986, Stephen H. Friend with his team isolated the first tumor suppressor gene – "Rb gene" – that was associated with an inherited (familial) form of cancer called retinoblastoma. With respect to breast and ovarian cancers in women, it was known that specific inherited mutations in the tumor suppressor gene "BRCA" increased the risks of these cancers as well as several other cancers in both men and women. Therefore, for more in-depth functional analysis, the tumor suppressor genes BRCA1 and BRCA2 were cloned in 1994 and 1995, respectively.

The tremendous expansion in the field of cancer biology subsequently led to the advancement of early cancer detection techniques, treatment, and prevention arenas. During the late 1990s, scientists were more interested in developing drugs that targeted the key features of cancer cells – proliferation, apoptosis, and angiogenesis. From 1996 to 2000, the FDA approved drugs like anastrozole (an aromatase inhibitor that blocks the production of estrogen in the estrogen receptor-positive advanced breast cancer women in postmenopausal stage), rituximab (a monoclonal antibody used in patients with treatment-resistant, low-grade, or follicular B-cell non-Hodgkin lymphoma (NHL)), and trastuzumab (a monoclonal antibody that targets cancer cells showing overexpression of HER2 protein, for the treatment of HER2-positive early-stage and metastatic breast cancer).

In 2000, Douglas Hanahan and Robert Weinberg published an influential peer-reviewed article named "The Hallmarks of Cancer" (Hanahan and Weinberg 2000), which was further updated in 2011 (Hanahan and Weinberg 2011). These hallmarks signified the common traits that differently govern the transformation of normal cells toward the cancerous form. Herein, the authors have also described how these traits are linked to the most common signaling pathways that get altered in the state of cancer (as elaborated in Sect. 1.3).

In 2001, the drug imatinib mesylate (sold under the brand named Gleevec by Novartis Company) was launched in trial form for the treatment of chronic myelogenous leukemia (CML). It targeted a unique protein produced by the Philadelphia chromosome and is effective in stopping the growth of cancer cells in case of

gastrointestinal stromal tumors (GIST). The Gleevec trial was one of the most successful trials in the history of cancer research. With the completion of the Human Genome Project in 2003, the entire sequenced map of human DNA became available for the cancer researchers, and this facilitated the exploration of the genomic path of cancer. In the same year, it was shown that reducing the production of certain hormones can help culminate the risk of developing a particular form of cancer. For example, in 2003, it was found that the drug finasteride assisted in lowering the risk toward prostate cancer in men by about 25%, as it could significantly reduce the production of male hormones in the body. Concurrently, in 2006, the antiestrogen drug raloxifene was shown to reduce the risk of developing breast cancer in postmenopausal women as well as serious side effects of the previously introduced drug tamoxifen. In this way, hormonal therapy was molded, and chemistry was tweaked in every possible manner to introduce different combination of drugs so as to regulate the predisposition toward specific forms of cancer. In the same year, FDA approved Gardasil – the first cancer prophylactic vaccine that protected against infection by two types of human papillomaviruses (HPV-16 and HPV-18), the etiologic agent and major cause of cervical cancer. Subsequently, in 2009, a second vaccine – Cervarix – was introduced for cervical cancer. In 2010, the first immunotherapy-based cancer treatment vaccine, sipuleucel-T, was approved by the FDA. This vaccine is made by using a patient's own dendritic cells and is used for the treatment of metastatic prostate cancer, in cases where the patient no longer responds to hormonal therapy. The fundamental thought of using immunotherapy for treatment of cancer was conceived by the great surgeon Stephen A. Rosenberg. This treatment was meant to enhance the activity of one's own immune system to prevent the spread of cancer by encouraging the destruction of tumors. In the same lines, FDA permitted the use of the monoclonal antibody, ipilimumab (IPI), for the treatment of advanced or metastatic melanoma in 2011. To harness the capability of the human immune system, IPI was designed in a way that it stimulated the immune system by binding to the human T cell, targeted them toward the cancerous cells, and inhibited their unwanted proliferation by increasing the intensity of immune responses. In 2013, the FDA approved an antibody-drug conjugate named adotrastuzumab emtansine (T-DM1). This immunotoxin is made by chemically linking the monoclonal antibody trastuzumab to the cytotoxic agent mertansine. It works by inhibiting the cell proliferation in HER2-positive breast cancer patients by blocking the formation of microtubules. With the advent of the Cancer Genome Atlas (TCGA) project in 2006 (an effort funded by the US government), researchers were able to analyze the DNA and mark the diversity of the cancer cells by identifying the molecular changes in more than 30 types of human cancers. The project aimed at focusing on the genomic characterization and sequencing of different tumor types by using massive sampling and creating a data set that enables researchers to improve the early stages of detection, treatment, and prevention in case of different cancers. Based on differing tumor characteristics, they were able to identify that gastric (stomach) cancer actually includes four different diseases. The findings from

the TCGA project will help in simplifying our understanding of the incredibly complicated cancer genome. These discoveries will also help in delineating the rapid evolution of cancer cells through a new classification system based on their genomic variability and molecular abnormalities.

Besides the discoveries mentioned in this section, there were various other studies in the field of cancer biology that have been vividly and elaborately covered in different chapters of this book.

1.3 Hallmarks of Cancer

In the paper- "The Hallmarks of Cancer," Douglas Hanahan and Robert Weinberg have studied different signaling circuits that together control the overall sustenance of a particular cell. It also describes how slight abruption in any single part of this cellular machinery can derail the governance of molecular controls and lead to cancer. When a normal cell overcomes these molecular restrictions that oversee the stringent functioning of different cellular signaling pathways, it transforms to a cancerous state. The general traits that are dominated in this state have been divided into three categories: the acquired capabilities, the enabling characteristics, and the emerging hallmarks (Hanahan and Weinberg 2000).

In the paper published in 2000, these researchers described six different acquired capabilities that have been observed to be common in a number of cancer genotypes. These included self-sufficiency in growth signals, insensitivity to antigrowth signals, tissue invasion and metastasis, sustained angiogenesis, limitless replicative potential, and evasion of apoptosis (Fig. 1.2). Manifestation of these six essential alterations in cell physiology during the course of multistep tumorigenesis can overall dictate the fate of a cell toward tumorigenesis. However, over a decade later, as the complexity of tumor cell biology started to unravel with the prospect of the contributions of "tumor microenvironment" toward tumorigenesis, the review article was updated with four more hallmarks of cancer in 2011 (Hanahan and Weinberg 2011). The acquisition of the earlier six hallmarks is made possible by the two enabling characteristics: genomic instability and random mutations and tumor-promoting inflammation. Two other attributes of cancer cells that are considered to be essential for the development of cancer cells include reprogramming of cellular energy metabolism and evasion from the attack of immune cells and avoiding immune destruction (Fig. 1.2). There have been various signaling pathways that have been extensively studied with respect to these hallmarks, and various cancer case studies have led to the identification of important molecules that are involved in cancer progression (Hanahan and Weinberg 2011).

Thus, the basic researches based on these hallmarks of cancer have globally expanded the pool of knowledge available on tumorigenesis. This, in turn, has greatly enhanced the development of novel targeted drug therapy that could interfere with each of these well-known and unique traits of cancer cells.

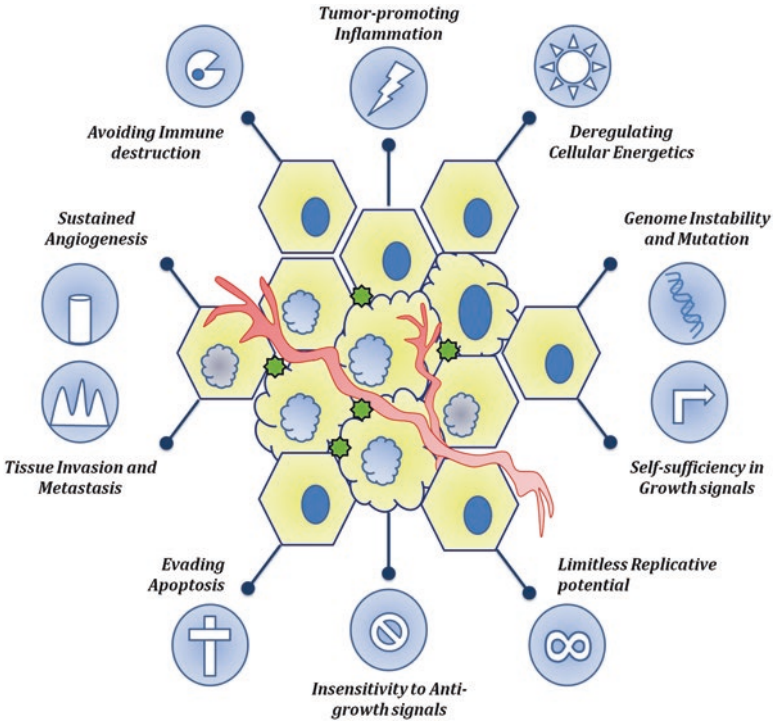

Fig. 1.2 Hallmarks of cancer

1.4 Conclusion

Over the years, as our knowledge in the field of cancer biology expanded, the revelation of various factors involved in cancer progression or suppression clarified our understanding of the intricate network and communication involved in case of different cancers. To a greater extent, it has led to the advancement in the field of technology that is now required to decipher the involvement of each of the cellular signaling network in the progression of different cancer phenotypes, their early detection, and subsequent manipulation of the treatment regimen for increasing the survival rates.

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Emerging Peptide-Based Technologies in Cancer Therapy

2

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Abstract

Peptides have emerged as powerful platform technologies to engineer effective therapies against cancer. Peptides play significant role as clinically important molecules in developing next-generation cancer therapeutics by targeting signalling pathways, cell cycle, tumour suppressor protein, transcription factor, ion channels, several G-protein-coupled receptors and matrix metalloproteinases MMP 2 and MMP 9. Peptide-based cancer vaccines also hold huge promise in cancer treatment. Peptide-based biomarkers have immense potential to facilitate the early detection of cancer and thus increase the lifespan of cancer patients. Additionally, peptides also act as safe clinically suitable molecular medicine transporters for cancer treatment. Cell-penetrating cationic peptides, lipopeptides, amphipathic peptides and mini-proteins facilitate nucleic acid-based drug delivery and can be

This book chapter is dedicated to Professor P. Balaram on his 70th birthday.

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exploited for developing siRNA-based nanotherapeutics and combination therapy against cancer. Peptide-based medicines and molecular transporters play significant role in managing adaptive chemotherapeutic drug resistance and metastasis against cancer. Here we have discussed the current status and emerging opportunities of peptides as molecular medicines and molecular transporters.

Keywords
Peptides · Cancer · Drug resistance · Metastasis · Peptide-based therapeutics · Cell-penetrating peptides

Peptides represent unique class of "molecular medicines" which are relatively clinically safe, potent and selective. This chapter highlights peptide-based cancer drugs and also peptide-based molecular transporters for delivering nucleic acid- and small molecule-based anticancer drugs. Cancer therapy has seen a giant leap of advancement in recent years, and the role of peptides is an indispensable part of this journey. At present, there are about 60 approved peptide-based drugs with an annual sale of around 13 billion dollars (Thundimadathil 2012). Some of the important peptide-based drugs for treating cancer are listed in Table 2.1 (Thundimadathil 2012). The number of peptide-based drugs entering in clinical trial has increased drastically from 1.2 per year in the 1970s to 16.8 per year in the 2000s (Borghouts et al. 2005). Like all other therapies, peptide-based therapeutics have their own advantages and disadvantages. High-end advantages include high potency of action, high target specificity and selectivity, wide range of targets, low toxicity, lesser side effects and lesser accumulation in tissues. On the other hand, some of the therapeutic peptides have metabolic instability, protease instability, poor membrane permeability and poor solubility and cannot be administered orally (Craik et al. 2013). Many strategies like cyclization, blocking C- and N-terminus, use of D-amino acids or unnatural amino acids and PEGylation are employed to make peptides protease resistant and increase their half-lives (Roberts et al. 2012; Schellenberger et al. 2009).

Peptides can be broadly classified mainly in the following three categories: (a) peptides for diagnosis of cancer, (b) cell-penetrating cationic and amphipathic peptides as molecular transporters and (c) peptides as anticancer drug. We will briefly discuss about them in the following sections.

2.1 Peptides in Diagnosis of Cancer

Peptide-based biomarkers can facilitate the early detection of cancer. Xiao et al. has summarized the peptides which can be used as biomarkers for the early detection of cancer (Xiao et al. 2015). Peptides uMMP-2 and RGS6 can be used to detect pancreatic cancer (Roy et al. 2014; Jiang et al. 2014). HNP1-3 and serum C peptide are present in higher amounts in colorectal cancer (Albrethsen et al. 2006; Comstock et al. 2014). Linear peptide antigen derived from ANXA1

Table 2.1 FDA-approved peptide-based anticancer drugs

Sl no.	Name of drug	Brand names	Sequence	Used for the types of cancer	Reference
1	Buserelin	Bigonist	Pyr-His-Trp-Ser-Tyr-D-Ser(OtBu)-Leu-Arg-Pro-NHEt (or N-ethyl-prolinamide), acetate; Pyr = pyroglutamic acid	Prostrate and breast	Sangeetha et al. (2019), Qayum et al. (1990)
2	Degarelix acetate	Firmagon	Ac-D-2Nal-D-4-chloroPhe-D-3-(3′-pyridyl)Ala-Ser-4-aminoPhe(L-hydroorotyl)-D-4-aminoPhe(carbamoyl)-Leu-isopropylLys-Pro-D-Ala-NH2, acetate	Prostrate	Sangeetha et al. (2019), Klotz (2009)
3	Goserelin	Zoladex	Pyr-His-Trp-Ser-Tyr-D-Ser(OtBu)-Leu-Arg-Pro-AzGly-NH_2, acetate [or [D-Ser(OtBu)6,AzGly10]GnRH, acetate] Pyr = pyroglutamic acid	Prostrate and breast	Sangeetha et al. (2019), Bolla et al. (1997)
4	Lanreotide acetate	Somatuline Depot	Cyclic lanreotide (sequence NH_2–(D) Naph-Cys–Tyr–(D)Trp–Lys–Val–Cys–Thr–$CONH_2$; BIM 23014C)	Gastroenteropancreatic neuroendocrine tumours (GEP-NETs)	Pouget et al. (2007)
5	Octreotide	Sandostatin	H-D-Phe-Cys(1)-Phe-D-Trp-Lys-Thr-Cys(1)-Thr-ol; both the Cys(1), Cysteine residues form intramolecular disulfide bond	Carcinoid (neuroendocrine) tumours	Craik et al. (2013), PubChem CID: 448601
6	Leuprolide	Eligard, Enantone, Leuplin, LeuProMaxx	Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHEt; Pyr = pyroglutamic acid	Prostrate	Thundimadathil (2012), Higano et al. (1996)

(H-FNTILTTRSYPQLRRVFQKYTLIRIMVSRSEID-OH) and peptides generated from variable part of antibodies are higher in patients with lung cancer (Wang et al. 2014c; De Costa et al. 2014). HCBP-1 is expressed in lung cancer stem cells (Wang et al. 2014a). Urinary matrix metalloprotease uMMP-2 and urinary tissue inhibitor of metalloprotease uTIMP-1 can be used to detect pancreatic cancer (Roy et al. 2014). Inhibitory cytokine 1 MIC-1/GDF15 is overexpressed in pancreatic duct adenocarcinoma tissues (Wang et al. 2014d). EN2 and mitochondrial uncoupling protein 2 are overexpressed in prostate cancer (Mcgrath et al. 2013; Li et al. 2013). HER-2 and carcinoembryonic antigen mucin1 have the potential to be used as biomarker for the early detection of breast cancer (Boku 2014; Zanetti et al. 2011).

2.2 Cell-Penetrating Cationic and Amphipathic Peptides as Molecular Transporters

Cell-penetrating peptides (CPPs) or peptide transduction domain (PTD) has gained extensive attention for their abilities of efficient intracellular drug delivery, especially nucleic acid-based therapeutics (Copolovici et al. 2014; Guidotti et al. 2017; Raucher and Ryu 2015; Zhang et al. 2015a). Cell-penetrating peptides have played a significant role in developing nucleic acid-based therapeutics. Small interfering RNAs or siRNAs are 21 to 25 base-paired double-stranded RNAs. siRNAs induce gene silencing by targeting complementary mRNA and degrading it. siRNAs have potential to target undruggable sites. Delivery is the critical problem for developing siRNA-based therapeutics. Naked siRNAs are easily degraded by nucleases. They also cannot easily cross the negatively charged cell membrane due to its anionic nature. Cationic peptide-encased siRNA has shown proteolytic stability, serum stability and functional delivery of siRNA and intracellular release of siRNA from peptide-siRNA complexes (Dutta et al. 2015; Biswas et al. 2019).

2.2.1 Structure of Cell-Penetrating Peptides

CPPs are synthetic or nature-derived peptides that are having 5–35 amino acid residues. CPPs were first identified by Frank and Pabo in 1988 (Rizzuti et al. 2015). They observed that activator protein of HIV-1 (TAT) could traverse through the cell membrane. After the initial discovery of TAT, research on short peptide-based CPPs, either derived from natural sources or synthesized, have grown rapidly (Joliot et al. 1991). The cellular uptake of cell-penetrating peptides relies on their sequences, structures, concentrations and cell lines used for the study (Duchardt et al. 2007; Yamada et al. 2014). Based on the sequences, cell-penetrating peptides can be of two types: cationic cell-penetrating peptides and amphipathic cell-penetrating peptides.

Cationic Cell-Penetrating Peptides Cationic cell-penetrating peptides contain positively charged amino acid residues comprising of a short stretch of arginine or lysine or histidine residues. The most widely used CPPs are TAT peptide which is

derived from HIV-1 protein, penetratin, an antennapedia homeodomain-derived peptide and nona-arginine sequence (Ren et al. 2012). Arginine-rich sequences, containing eight or nine arginine residues, exhibit enhanced cellular uptake (Herce et al. 2014). Histidine (His), lysine (Lys) and arginine (Arg) are having side chain pKa values of 6.04, 10.54 and 12.48, respectively. Histidine is not charged at extracellular matrix having pH 7.4. Therefore, Lys and Arg are more effective to engineer cell-penetrating peptides (Lehto et al. 2010). Between Lys and Arg, Arg is bidentate due to the presence of planar Y-shaped guanidinium moiety that can form a bidentate hydrogen bond with cell surface anionic moieties such as phosphate (PO_4^{3-}), carbonate (CO_3^{2-}) and sulphate (SO_4^{2-}) (Rothbard et al. 2002). It is well documented in the literature that spacing between two adjacent arginine residues in cell-penetrating peptides facilitates cellular uptake (Lehto et al. 2010; Dutta et al. 2015). The uptake efficiency of cationic peptides, having (RXR)4 template, where X is glycine, β-alanine, 4-aminobutyric acid, 6-aminocaproic acid or 8-aminocaprylic acid, was examined. The spacer having 6-aminocaproic acid exhibited maximum efficacy, inferring that the cellular uptake increases with increased spacing between two adjacent arginine residues (Lehto et al. 2010; Rothbard et al. 2002). This indicates that minimizing the adjacent Arg-Arg repulsion is necessary for efficient uptake (Rothbard et al. 2002; Dutta et al. 2015). Additionally, lipidic modification of cationic peptide sequences improves the cell permeation (Lehto et al. 2010; Dutta et al. 2015; Biswas et al. 2019). Dutta et al. have reported doubly stearylated peptide having Arg-Sar-Arg sequences as efficient molecular transporter (Dutta et al. 2015). The spatial location of cationic residues and the structural rigidity of peptide can also affect their cellular internalization (Lättig-Tünnemann et al. 2011). Cellular transduction efficiencies of peptides, linear TAT, cyclic TAT and linear and cyclic decaarginine (R10) were compared, and it was shown that cyclic structure has enhanced the transduction efficiency due to the proteolytic stability (Lättig-Tünnemann et al. 2011). Cyclization rigidifies the spatial location of arginine side chain, reduces conformational possibilities and reduces adjacent Arg-Arg repulsion, enhances the proteolytic stability of peptides and stability of peptide-siRNA complexes and consequently enhances transduction efficiency (Lättig-Tünnemann et al. 2011; Dutta et al. 2015). It has been reported that cyclic R9 exhibited 40% increased cellular internalization than its linear counterpart (linear R9) (Traboulsi et al. 2015). The effect of exocyclic and endocyclic position of arginine residues on cyclic polyarginine peptide has also been examined (Traboulsi et al. 2015). Cyclic R4-R3 (endocyclic = R4, exocyclic = R3) shows higher cellular penetration than cyclic R7 (endocyclic = R7) analogue, and cyclic R4-R3 shows equal penetration ability like linear R9 (Traboulsi et al. 2015). Cyclic and bicyclic approaches are good strategies for providing stability to the peptide-siRNA complexes. Professor Roger Tsien's research group has shown that activatable cell-penetrating peptides (ACPPs) linked to nanoparticles can be used as dual probes for in vivo fluorescence and MR imaging of matrix metalloproteinase (Olson et al. 2010). ACPPs contain short polycations and polyanions, which are attached via protease-cleavable linker. MMP 2 and MMP 9 protease which are predominantly present in metastatic niche cleave this linker having the sequence PLGLAG. This in turn releases the polycationic part and its associated cargo to be uptaken by cells (Olson et al. 2010). Such dual probes can

be used for high-resolution imaging in malignancy and metastasis. Fluorophore-tagged activatable cell-penetrating peptides facilitate residual tumour removal during surgery and improve the survival of cancer patients (Nguyen et al. 2010; Metildi et al. 2014).

Amphipathic Cell-Penetrating Peptides Amphiphilic peptides show enhanced cellular internalization compared to oligocationic peptides. The cationic part of amphiphilic peptides binds with negatively charged cargo, whereas hydrophobic part interacts with the lipid bilayer (Dutta et al. 2015; Biswas et al. 2019). Penetratin is an amphiphilic helical peptide, identified from Drosophila antennapedia protein homeodomain, and it adopts various secondary structures depending on the environments (Magzoub et al. 2002). It takes unstructured conformation in presence of membrane bilayer and β-sheet structure in the cytoplasm. Penetratin forms complexes with siRNA at a low molar ratio (10:1), but due to endosomal entrapment of peptide-siRNA complex, it does not show any silencing in vitro (Lundberg et al. 2007). Interestingly, penetratin has an ability to deliver exogenous siRNA to the cytoplasm when covalently attached with siRNA (Davidson 2004; Muratovska and Eccles 2004). CADY is a 20-residue-long amphiphilic α-helical peptide which binds with siRNA with high affinity through electrostatic and hydrophobic interactions and showed significant knockdown of the target gene (Tai and Gao 2017). Amphipathic peptide, KALA, shows pH-dependent conformation changes from random coil structure to the α-helical structure as pH changes from 5.0 to 7.5 (Wyman et al. 1997). At physiological pH, it shows helical nature where one face displays hydrophilic lysine residues and other face displays hydrophobic residues. KALA-siRNA complex transfected anti-GFP and anti-VEGF siRNA in tumour cells (Lee et al. 2007; Mok and Park 2008). Considering the intracellular release of siRNA from peptide-siRNA complexes, amphipathic peptides having less cationic charges will be more effective molecular transporter compared to oligocationic molecular transporters.

2.2.2 Polypeptide- and Protein-Based Transporters

Several groups have examined the use of larger polypeptides or proteins having both less and excess cationic charges to transfect several hard-to-transfect cell lines. Professor Alanna Schepartz's group has used the 36-residue avian pancreatic polypeptide having well-folded α-helix and polyproline II (PPII) as template for engineering minimally cationic cell-permeable miniature proteins (Smith et al. 2008). Several peptides were designed which have different numbers of arginine residues incorporated into the solvent-exposed side of the helices. Interestingly, enhanced cell permeability was observed with peptide having two arginine residues (Smith et al. 2008). Professor Dowdy's group at UCSD has designed a 65-residue polypeptide conjugating TAT peptide transduction domain (PTD) with a single dsRNA-binding domain (DRBD) and demonstrated that PTD-DRBD fusion domain can show

efficient siRNA delivery to human umbilical vein endothelial cells (HUVEC), T cells and human embryonic stem cells (hESCs), which are considered as hard-to-transfect cell lines (Eguchi et al. 2009). Octamer of ribonucleoprotein is also used for targeted siRNA delivery against polo-like kinase 1 gene in prostate cancer model (Tai et al. 2018). Supercharged GFP protein (+36 GFP) was used for siRNA and DNA transfection in mammalian cells (Mcnaughton et al. 2009). +36 GFP has both protease stability and serum stability and extends the serum half-life of siRNA and DNA. +36 GFP has demonstrated to deliver siRNA into a variety of mammalian cells, which is even resistant to cationic lipid-based transfection reagent (Mcnaughton et al. 2009). The above examples demonstrate the use of cell-specific transfection reagent depending on the membrane composition of a particular cell line.

2.2.3 Mechanism of CPP-Cargo Internalization Pathways

The cellular uptake pathways of CPPs depend on the physiochemical properties, concentration of CPPs, concentration of CPP-siRNA complexes and types of cell lines used (Duchardt et al. 2007). Internalization pathway for cellular uptake could be ATP independent (translocation or direct penetration) or ATP dependent (endocytosis) (Duchardt et al. 2007).

Direct Penetration Cellular uptake of certain arginine-rich peptides follows ATP-independent direct internalization pathway (Mandal et al. 2011; Duchardt et al. 2007). Direct internalization of cell-penetrating peptides may depend on peptide sequence and concentrations (Duchardt et al. 2007). Cationic peptides and lipopeptides interact with negatively charged cell surface carboxylates, phosphates, glycosylates, etc. followed by cellular internalization by transient pore formation. Along with transient pore formation, the internalization of lipopeptides also happens due to membrane destabilization by lipidic moiety (Madani et al. 2011). Direct penetration follows several pathways like transient pore formation model, carpet-like model or inverted-micelle mechanism (Madani et al. 2011).

Transient pore formation model involves toroidal pores or barrel-stave pores. In toroidal pore model, CPPs attach to the outer face of the membrane, and after reaching certain threshold concentration, cellular entry happens through membrane pores. In barrel-stave pore model, CPPs assume a helical conformation, and its hydrophobic face aligns with the lipid core of the membrane (Yamada et al. 2014). According to the carpet like model, a positively charged moiety of lytic peptide and negatively charged phospholipid binds together which shape like a carpet and, subsequently, reaches a certain concentration followed by local membrane destabilization, leading to internalization of CPPs and CPP-cargo complexes (Hong and Su 2011). In inverted micelle mechanism, positively charged residues like arginine or lysine primarily bind to negatively charged phosphate group of lipids and merge together to convert into micelle which internalizes CPPs (Trabulo et al. 2010).

Endocytosis Endocytosis is an energy-dependent process of cargo internalization. It includes various different pathways: clathrin- or caveolae-mediated endocytosis and macropinocytosis (Madani et al. 2011). The internalization depends upon the size, shape and physicochemical properties of peptide-cargo complexes. To identify the different endocytosis pathways, various endocytosis inhibitors have been reported (Biswas et al. 2019; Dutta et al. 2015; Duchardt et al. 2007). The major obstacle of siRNA therapy is the entrapment of cargo inside the endosome that limits the successful release of cargo in cytosol.

2.2.4 Endosomal Escape

Conjugation of pH-sensitive moieties to carrier peptides facilitates the rupture of endosomal membrane and cytosolic delivery of entrapped cargo (Boussif et al. 1995). The pH-responsive agent exhibits buffering capacity at lower endosomal pH which is termed as "proton sponge effect". Proton sponge describes the protonation of amines and induces an influx of ions and water into endosomal vesicles leading to osmotic swelling, rupture of endosomal membrane and escaping of entrapped molecules (Boussif et al. 1995). Histidine residue and polyethylenimine (PEI) (Kwon et al. 2008; Varkouhi et al. 2011) absorb proton in endosome. To balance this proton influx, outside chloride ions and water molecules enter inside the endosomal vesicles. This causes osmotic swelling and rupture of the endosomal vesicles with the release of cargo molecules to the cytosol (Biswas et al. 2019; Varkouhi et al. 2011; Dutta et al. 2015; Kichler et al. 2003; Hatefi et al. 2006; Singh et al. 2004; Kichler et al. 2006).

2.2.5 Protein Corona and PEGylation

The extravagant use of nanocarriers in biology faces the complexity due to less understanding about the interaction of nanoparticles (NPs) with biological surroundings (Saptarshi et al. 2013). When the nanoparticles are introduced into biological fluids, proteins are adsorbed onto the nanoparticles, and NP-protein complexes are generated (Nguyen and Lee 2017). The adsorption of proteins on nanoparticles depends on physicochemical properties of nanoparticles, e.g. size, charge of the surface, functionality and temperature and pH of the biological media (Saptarshi et al. 2013).

Proteins with large quantities and low affinity are adsorbed first onto nanoparticle surface (formation of soft corona) and then subsequently replaced by higher-affinity proteins (forming hard corona). This is known as "Vroman effect" (Turbill et al. 1996). Nanoparticles are furnished with different functional moieties for selective drug delivery to targeted sites. To circumvent this situation, polyethylene glycol (PEG) is attached with nanoparticle to form hydrophilic "stealth". This PEG moiety facilitates the targeted delivery of the nanoparticles (Corbo et al. 2016). PEGylation improves the solubility of nanoparticle, hinders adsorption of proteins on the surface of nanoparticle and enhances half-life of drugs in the bloodstream (Banerjee et al.

2012). The PEGylation of liposomal doxorubicin (Doxil) has exhibited enhanced lifetime of the drug from minutes to hours (Gibson et al. 2013). It shows a half-life ~100 times longer than free doxorubicin (Suk et al. 2016). PEGylation can be implemented in various cases, e.g. proteins, antibodies, cytokines, etc., to improve protein stability, reduce immunogenicity and prevent rapid renal clearance. Therefore, it allows a reduced dosing frequency. Hence, PEGylation has improved the drug formulation. PEGylation maintains its ability to open the cellular tight junctions and enhance molecular permeability (Laksitorini et al. 2014). It also reduces the toxicity of some biomolecules, e.g. chitosan (Casettari et al. 2010). In spite of its landmark success, the non-biodegradable nature of PEG may become a limiting factor for its application as next-generation therapeutics (Pisal et al. 2010).

2.3 Cell Surface Receptor Peptides for Active Targeting

Tumour-targeting peptides bind to cell surface receptors upregulated in cancer cells. In this section, we have listed cancer-associated upregulated receptors and tumour-specific sequences used in targeted nanomedicine and tested in in vivo models. Gray and Brown have reported an elaborate list of receptor-targeting peptide sequences (Table 2.2) (Gray and Brown 2013). Peptide RGD (Arg-Gly-Asp) recognizes and binds to integrin $\alpha\nu\beta3$ and $\alpha\nu\beta5$ which are upregulated at the angiogenic blood vessels of malignant tumours (Wickham et al. 1993). This RGD peptide-conjugated drug carrier can be internalized in cells and can also be used as targeted drug carrier (Chen et al. 2012). ATN-161, a non-RGD pentamer-capped peptide (Ac-PHSCN-NH2), also binds to integrin and suppresses breast cancer growth and metastasis in vivo (Khalili et al. 2006). It is reported that CXCR4 (a chemokine receptor) is overexpressed in multiple types of cancer like lung cancer. LFC131, a pentapeptide has good affinity for CXCR4. So, LFC131 acts as a targeting molecule for anticancer drug like docetaxel or nanoparticle (Wang et al. 2015). Another peptide, NGR (Asn-Gly-Arg), binds to aminopeptidase N (APN), also called CD13 (Majhen et al. 2006), which is highly expressed by angiogenic blood vessels of tumours, pancreatic cancer and non-small cell lung carcinoma (Zhang et al. 2015b).

Table 2.2 Receptor-targeting peptide sequences (Gray and Brown 2013)

Sl no.	Cellular target (Receptor)	Peptide sequence
1	Met	YLFSVHWPPLKA
2	EGFR	YHWYGYTPQNVI (GE11)
3	VEGFR-3	**C**SDSWHYW**C**
4	HER2/ErbB2	WTGWCLNPEESTWGFCTGSF
		KCCYSL
		MARSGL
		MARAKE
		MSRTMS
5	CXCR4	FC131 [cyclo(-D-Tyr-Arg-Arg-Nal-Gly-)]; Nal: 3-(2-naphthyl)-alanine (Inokuchi et al. 2011)

NGR has been conjugated with carrier molecules to deliver siRNA and drugs like doxorubicin (Chen et al. 2010). Lyp-1, a 10-mer cyclic peptide, is a tumour-homing peptide which is cytotoxic in nature, reduces the number of lymphatic vessels and inhibits tumour growth in xenograft mice breast cancer model (Laakkonen et al. 2004). Targeted drug delivery requires overexpression of a target receptor in cancer cells and its accessibility on the tumour cell surface (Roveri et al. 2017). Therefore, the design of an optimal ligand-decorated nanocomplex should rely on the biological features and functions of the target receptor.

2.4 Peptides as Anticancer Drugs

Peptides have been extensively used as anticancer drugs. According to their mode of actions, they can be classified as follows: (a) membrane-active peptides in cancer therapy, (b) peptide-based inhibitors targeting signalling pathways, (c) cell cycle-arresting peptides, (d) peptides inducing cell death, (e) peptides targeting tumour suppressor proteins and transcription factors, (f) peptides targeting tumour microenvironment, (g) tumour pH- and temperature-responsive peptides, (h) peptides as immunomodulators and vaccines against cancer, (i) venom peptides as cancer drugs and (j) anti angiogenic peptides.

2.4.1 Membrane-Active Peptides in Cancer Therapy

The anticancer efficacy of natural ionophores, salinomycin, monensin and gramicidin A (gA) has recently been evaluated (Huczyński 2012). Rajasekaran and co-workers reported that gA shows anticancer effect against renal cell carcinoma in non-apoptotic pathway (David et al. 2014). Rao et al. has reported that gA and curcumin can be used as anticancer agents against drug-resistant cell lines HEK-293, ABCG2 cells and MCF7/FLV1 (482R) cells overexpressing ABCG2 (Rao et al. 2014). Recently, Chakraborty et al. have reported that gA and gA-inspired octapeptide, LD8, made of alternating L- and D-amino acid residues which exhibited cytotoxicity and apoptotic cell death against metastatic breast cancer cell line MDA-MB-231 (Chakraborty et al. 2018). The cytotoxicity was increased when doxorubicin (Dox) was loaded with gA and LD8. Interestingly Dox-loaded gA and Dox-loaded LD8 show apoptosis by different mechanisms (Chakraborty et al. 2018). This is probably the first report that gA can be used against metastatic breast cancer cell line.

AMPs like magainin and pleurocidin (isolated from *Xenopus laevis* and *Pleuronectes americanus*, respectively) can also act as anticancer drugs (Zasloff 1987; Cole et al. 1997). Magainin II is cytotoxic against human bladder cancer cells but did not show any significant effect on human or murine fibroblast cell line (Lehmann et al. 2006). Pleurocidin is cytotoxic to human breast cancer cell lines and murine mammary carcinoma cells but has no effect on human dermal fibroblast cell lines. Both the peptides disrupted the plasma membrane (Hilchie et al. 2011), and their selective specificity of action against cancerous cell line might have a significant aspect in cancer treatment. Buforin IIb (another AMP, isolated from

stomach of *Bufo bufo gargarizans*) (Park et al. 1996) showed cytotoxicity against HeLa (cervical carcinoma) cells and Jurkat (leukaemia) cells and also reduces the growth of human lung cancer in mice xenograft model (Lee et al. 2008a). A peptide DP1 where antimicrobial peptide $(KLAKLAK)_2$ is fused to peptide transduction domain (PTD-5) induces apoptosis by mitochondrial swelling and disruption of mitochondrial membrane (Mai et al. 2001). Table 2.3 lists several AMPs used in cancer treatment.

2.4.2 Peptides Targeting Signalling Pathways

Peptides targeting signalling pathways can be exploited for cancer treatment also for developing combination therapy against cancer. Peptides targeting MAPK pathway are highly beneficial for cancer therapy, since MAPK pathway is constitutively activated in pancreatic, breast, colon, lung, ovarian and kidney cancer (Hoshino et al. 1999). MAPK pathway has three subfamilies, and their effector molecules are ERK, JNK and p38. Cardiac natriuretic peptides like ANP, LANP, vessel dilator peptide and kaliuretic peptide inhibit the activation of ERK1/2 in human prostate adenocarcinoma and pancreatic cancer cells (Sun et al. 2007a). LANP and ANP showed 80–90% inhibition of MEK1/2 in human prostate adenocarcinoma cell line (Sun et al. 2007b). JIP (JNK-interacting protein) inspired peptide JIP10 (PKRPTTLNLF) acts as JNK protein inhibitor. When peptide JIP10 is conjugated with TAT or R9, the migration of PyVMT cells get inhibited by selective inhibition of JNK2 protein. This TAT- or R9-conjugated JNK inhibits JNK2 activity and inhibits metastasis of murine mammary cancer cells in vivo (Kaoud et al. 2011). Tetrapeptides VWCS and FWCS inhibit p38α and also inhibit proliferation of human oral cancer cell lines (Gill et al. 2013, 2014). Table 2.4 lists some peptide sequences used for targeting signalling pathways for cancer treatment.

2.4.3 Cell Cycle-Arresting Peptides for Cancer Treatment

p16 is a tumour suppressor protein, and it inhibits the progression of cells from G1 to S phase by binding with Cdk4/6 and inhibits cyclin D to bind to it. Mutated p16 proteins are found in many types of cancers like breast, cervix, skin, colon, prostate and brain. Transfection of p16 gene causes apoptosis in human brain, prostate, lung and bladder cancer cell lines (Marqus et al. 2017). Fahraeus et al. (Fåhraeus et al. 1996) reported a 20-amino acid synthetic peptide of p16 (84–103 amino acids) (DAAREGFLDTLVVLHRAGAR) arrests HaCat (a primary epidermal keratinocyte cell line), MCF-7 (a breast cancer cell line), MRC-5 (normal lung fibroblast cell line), HT-29 (cell line from colorectal adenocarcinoma) and 3T3 (mouse fibroblast cell line) cell lines from entering S phase (Fåhraeus et al. 1998). p21 is also a CDK inhibitor. C-terminal part of peptide p21 mainly arrests cell cycle at G1 like p16 (Ball et al. 1997) and inhibits DNA repair in HeLa cells. Peptide p21 when fused with penetratin induces necrosis in human lymphoma cells (Marqus et al. 2017). Doxorubicin (Dox)-loaded gA caused cell cycle arrest at S phase in metastatic

Table 2.3 Membrane-active peptide sequences used for cancer therapy

Sl no.	Peptide	Peptide sequence		Cancer type	Reference
1	Gramicidin A (gA)	Formyl-^{L}Val-Gly-^{L}Ala-^{D}Leu-^{L}Ala-^{D}Val-^{L}Val-^{D}Val-^{L}Trp-^{D}Leu-^{L}Trp-^{D}Leu-^{L}Trp-^{D}Leu-^{L}Trp-ethanolamine		Kidney, breast	David et al. (2014), Chakraborty et al. (2018)
2	Magainin II	GIGKFLHSAKKFGKAFVGEIMNS		Bladder cancer	Lehmann et al. (2006), PubChem CID:16166807
3	Pleurocidin	NRC-03	GRRKRKWLRRIGKGVKIIGGAALDHL-NH_2	Breast	Hilchie et al. (2011)
		NRC-07	RWGKWFKKATHVGKHVGKAALTAYL-NH_2		
4	Buforin IIb	(RAGLQFPVG[RLLR]$_3$)		Cervix	Lee et al. (2008a)
5	LD8	Boc-^{L}Ala-^{D}Val-^{L}Leu-^{D}Ala-^{L}Val-^{D}Ala-^{L}Leu-^{D}Trp-OMe		Breast	Chakraborty et al. (2018)
6	PTD-5 conjugated DP1	RRQRRTSKLMKRGGKLAKLAKKLAKLAK		Head and neck tumours	Mai et al. (2001)

Table 2.4 Peptide sequences for targeting signalling pathways for cancer treatment

Sl no.	Peptide	Peptide sequence	Targeted signalling molecule	Reference
1	ANP	H-Ser-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Met-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr-OH trifluoroacetate salt (Disulfide bond)	ERK1/2 MEK1/2	Sun et al. (2007a), Sun et al. (2007b), BACHEM Catalog Number H-2095
2	LANP	npmynavsnadlmdfknlldhleekmpled	ERK1/2 MEK1/2	Oikawa et al. (1984), Sun et al. (2007a), International Publication Number: WO 2012/138429 Al
3	Vessel dilator peptide	EVVPPQVLSEPNEEAGAALSPLPEVPPWTGEVSPAQR	ERK1/2	Oikawa et al. (1984), Sun et al. (2007a), Patent No: EP2678002A2
4	VWCS	VWCS	p38α	Gill et al. (2013)
5	FWCS	FWCS	p38α	Gill et al. (2014)

breast cancer cell line MDA-MB-231 indicating inhibition of DNA synthesis and subsequent entry into mitosis (Chakraborty et al. 2018). Interestingly, when Dox was loaded with peptide LD8 (Boc-^{L}Ala-^{D}Val-^{L}Leu-^{D}Ala-^{L}Val-^{D}Ala-^{L}Leu-^{D}Trp-OMe), a gA-inspired peptide, caused G2/M phase cell cycle arrest against the same cell line suggesting DNA damage after treatment (Chakraborty et al. 2018).

2.4.4 Peptides Inducing Cell Death

Bcl 2 family of proteins are either pro-apoptotic or anti-apoptotic. These proteins contain BH1–BH4 domains. All the four domains are present in anti-apoptotic proteins. Pro-apoptotic proteins are either multidomain (consisting of all domains except BH4) or BH3 only. Bim is a peptide with BH3 domain only. Conjugation of TAT with BH3 domain derived from Bim causes apoptosis in mouse T-cell lymphoma and pancreatic cancer cells. Apoptosis is enhanced if these cells are additionally exposed to radiation (Kashiwagi et al. 2007). Membrane pore-forming domain of Bax, called poropeptide-Bax (106–134-amino acid segment), causes the release of cytochrome C (Cyt C) in the case of malignant melanoma cell line SK-MEL-28 leading to apoptosis (Valero et al. 2011). N-terminal region of poropeptide-Bax conjugated with octa-arginine (R8) induces cell death in HeLa cells (Valero et al. 2011). CT20 peptide (173–192 amino acids) derived from C-terminal of Bax, conjugated with nanoparticle, was found to be cytotoxic against highly metastatic breast cancer cell line MDA-MB-231. Interestingly, it shows no toxicity against normal human breast epithelial cell line MCF-10A (Lee et al. 2014). Sequences of peptide inducing cell death are mentioned in Table 2.5.

2.4.5 Peptides Targeting Tumour Suppressor Proteins and Transcription Factors

p53 is a well-known tumour suppressor protein which accumulates in cell in case of DNA damage, and its accumulation causes apoptosis. Binding of mouse double minute 2 protein (MDM2) to p53 causes rapid degradation of p53. TIP (thioredoxin

Table 2.5 Peptide sequences for inducing cell death in cancer cells

Sl no.	Peptide	Peptide sequence	Cell line	Reference
1	TAT-Bim	Ac-rkkrr-orn-rrr-EIWIAQELRRIGDEFNAYYAR-OH; orn=Ornithin; r = ^{D}Arg; k = ^{D}Lys	mouse T-cell lymphoma, pancreatic cancer cells	Kashiwagi et al. (2007)
2	Bax [106–134]	NWGRVVALFYFASKLVLKAL STKVPELIR	Malignant Melanoma	Valero et al. (2011)
3	R8-Bax [106–134]	FITC-RRRRRRRRGNWGRVVALFYFA SKLVLKALCTKVPELIR	HeLa	Valero et al. (2011)
4	CT20	Ac-VTIFVAGVLTASLTIWKKMG-NH_2	MDA-MB-231	Lee et al. (2014)

Table 2.6 Peptide sequences for targeting transcription factors

Sl no.	Peptide	Peptide sequence	Target	Reference
1	PNC-27 (12–26)	PPLSQETFSDLWKLL	MDM2	Kanovsky et al. (2001)
2	PNC-21 (12–20)	PPLSQETFS	MDM2	Kanovsky et al. (2001)
3	PNC-28 (17–26)	ETFSDLWKLL	MDM2	Kanovsky et al. (2001)
4	Hl-F8A,S6A	NELKRAFAALRDQI	c-Myc	Draeger and Mullen (1994)
5	TAT-α HDM2	YGRKKRRQRRRG-QETFSDLWKLLP	HDM2	Harbour et al. (2002)

insert protein) derived from MDM2-binding region of p53 inhibits binding of p53 to MDM2 protein causing the accumulation of p53 and thereby causes apoptosis in SA1 osteosarcoma cells which overexpresses MDM2 protein (Wasylyk et al. 1999). PNC-27 (12–26 amino acids), PNC-21 (12–20 amino acids) and PNC-28 (17–26 amino acids) are synthesized from MDM2-binding region of p53. Interestingly, CPP conjugated to PNC-21, PNC-27 and PNC-28 showed cytotoxicity selectively against malignant cell lines but not to non-cancerous cell lines (Kanovsky et al. 2001). TAT-αHDM2 (a 12-amino acid peptide, derived from p53 which helps in binding to HDM2) inhibits the binding of p53 to HDM2 (human homologue of MDM2). The accumulated p53 activates apoptotic genes, promotes cell cycle arrest and induces apoptosis of U-2 OS, a human osteosarcoma cell line (Harbour et al. 2002). A 14-residue peptide (Hl-F8A,S6A) inhibits binding of transcription factor c-Myc to DNA (Draeger and Mullen 1994). Hl-F8A,S6A fused with penetratin inhibits the cell proliferation, promotes apoptosis of human breast cancer cell line MCF-7 and also blocks the action of c-Myc (Giorello et al. 1998). Sequences of some important peptides for targeting transcription factors are given in Table 2.6.

2.4.6 Peptides Targeting Tumour Microenvironment

Malignant tumour microenvironment constitutes of stromal cells and extracellular matrix with leaky blood vessels. Glycoprotein prosaposin (PSAP) is cleaved in endosome and released in the microenvironment. This stimulates the production of tumour suppressor protein thrombospondin-1 by macrophages which inhibits breast and lung cancer (Carvelli et al. 2015; Kang et al. 2009). A cyclic pentapeptide [c(DWLPK)] is developed from PSAP sequence, and it decreases the metastasis rate in xenograft murine model of ovarian cancer (Wang et al. 2016). CXCR4, a chemokine receptor, is responsible for epithelial-mesenchymal transition (EMT). Synthetic antagonist of CXCR4, also known as NT21MP (21 amino acids), shows decreased metastasis in breast cancer, reversal of EMT and override drug resistance in breast cancer (Yang et al. 2014). Another CXCR4 antagonist Nef-M1 (Bumpers et al. 2013; Katkoori et al. 2015) induces apoptosis and inhibits angiogenesis and metastasis in xenograft models (Bumpers et al. 2013, Katkoori et al. 2015). Another gold

Table 2.7 Sequences of peptides targeting tumour microenvironment

Sl no.	Peptide	Peptide sequence	Target protein	Reference
1	NT21MP	LGASWHRPDKCCLGYQKRPLP	CXCR4	Yang et al. (2017)
2	Nef-M1	NAACAWLEAQ	CXCR4	Mendes et al. (2018), Bumpers et al. (2013), Katkoori et al. (2015)
3	AARP	CTTHWGFTLC	MMP 2, MMP 9	Le Joncour and Laakkonen (2018)
4	Peptide R	Arg-Ala-[Cys-Arg-Phe-Phe-Cys]	CXCR4	Le Joncour and Laakkonen (2018), Mukhopadhyay et al. (2013)

standard antagonist of CXCR4, called peptide R, is successful in remodelling of tumour stroma and even had intracranial localization in xenograft model (Le Joncour and Laakkonen 2018; Mukhopadhyay et al. 2013). A peptide-peptide combination named AARP peptide inhibits MMP 2 and MMP 9. It also inhibits angiogenesis and metastasis (Le Joncour and Laakkonen 2018). Sequences of peptides targeting tumour microenvironment are given in Table 2.7.

2.4.7 Tumour pH- and Temperature-Responsive Peptides

The tumour microenvironment has a lower intracellular pH (pH 7.0–7.2) than normal physiological pH (7.4), while the extracellular pH is acidic (pH-6.8) (Zhang et al. 2010). The tumour environment is mild hyperthermic than normal tissues mainly due to dysregulated angiogenesis, and these characters are exploited while delivering drugs. A 36-residue peptide (pH-low insertion peptide (pHLIP)) (Hunt et al. 1997), derived from bacteriorhodopsin C helix, shows selective membrane affinity to cancer cell membrane having slightly acidic pH. Interestingly, at basic or neutral pH, this peptide loses its helical structure, and its affinity to membranes is reduced (Hunt et al. 1997). Due to this pH-sensitive behaviour, pHLIP has great prospect as targeted drug delivery in the future. A 42-residue leucine zipper peptide-lipid hybrid vesicle adopts coiled-coil structure, and it dissociates at slightly higher temperature > 40 °C. This dissociation in slightly higher temperature can be used to deliver drugs like doxorubicin which will only be released at hyperthermic tumour microenvironment (Al-Ahmady et al. 2012). Elastin-like peptide (ELP) is a repeated pentapeptide (VPGXG), where X is any amino acid except proline and is conjugated with a CPP. This biopolymer ELP is soluble at physiological temperature (37 C) but insoluble and forms aggregates at 40–42 °C (Bidwell III and Raucher 2010). ELP has been widely used as a delivery system for cancer therapy and has also shown to overcome drug resistance (Mackay et al. 2009; Bidwell et al. 2007). Table 2.8 lists a few such important peptides.

Table 2.8 Peptide sequences for targeting tumour pH and temperature

Sl no.	Peptide	Peptide sequence	Role	Reference
1	pHLIP	ACEQNPIYWARYADWLFTTPLLLLDLALLVDADET	pH sensitive	Le Joncour and Laakkonen (2018), Hunt et al. (1997)
2	Leucine zipper peptide	[VSSLESKVSSLESKVSKLESKKSKLESKVSKLESKVSSLESK]-NH_2	Temperature sensitive	Al-Ahmady et al. (2012)
3	Elastin-like peptide (ELP)	VPGXG, where X = any amino acid except proline	Temperature sensitive	Bidwell III and Raucher (2010)

2.4.8 Peptides as Immunomodulators and Vaccines Against Cancer

Tumour cells express some special antigens such as tumour-associated antigens (TAA) or neoantigen or mutation-derived antigens. This phenomenon is utilized in designing peptide vaccines by eliciting tumour-based immune response. Peptide vaccines are mainly consisting of TAA-derived cytotoxic T lymphocyte (CTL) epitopes, T-helper cell epitopes or dendritic cells loaded with TAA-derived peptides. Immunogenicity is improved by administering monoclonal antibodies (mAbs) against immunosuppressors like CTLA-4 and PD-1 proteins (Hirayama and Nishimura 2016). Engineered cystine-knot peptide (knottins) MCoTI-II (SGSDGGVCPKILKKCRRDSDCPGACICRGN-GYCG) can bind to CTLA-4 and can be used to treat metastatic melanoma (Maaß et al. 2015). Peptide RK-10 (GSGSGSTYLCGAISLAPKAQIKESL) binds to PD-L1, the ligand for PD-1, and can restore host T-cell function (Caldwell et al. 2017). Peptide antigens are generally 8–10 amino acids long with 2–3 residues acting as primary anchor to MHC-I and 2–3 residues binding to T-cell receptor (Gao and Jakobsen 2000; Cho and Celis 2009). Several peptide-based vaccines are in clinical trial and have yielded satisfactory results against pancreatic, gastric, prostate, breast, colorectal and lung cancers (Xiao et al. 2015). Some of the peptides used for designing peptide vaccines include Mucin-1, carcinoembryonic antigen, prostate-specific membrane antigen, HPV-16 E7 peptide (MLDLQPETT) and Ras oncoprotein peptide (Thundimadathil 2012). Sipuleucel-T was the first FDA-approved cancer vaccine for the treatment of castration-resistant prostate cancer patient (Cheever and Higano 2011). Cancer vaccine with epidermal growth factor variant III-derived peptide (EGFRvIII) has the potential to enhance survival of patients with glioblastoma or other types of tumours that express EGFRvIII (Li et al. 2010). Recent studies show great aspect for the prevention of cervical cancer by the aid of few peptide-based human papilloma virus vaccine (Trimble and Frazer 2009). Ishikawa et al. reported that LY6K (lymphocyte antigen 6 complex locus K), a typical cancer/test antigen, is expressed in 85% of observed gastric cancers (Ishikawa et al. 2014). Vaccination with LY6K-177 (LY6K-derived peptide) (RYCNLEGPPI) emulsified with Montanide ISA 51 (a mixture of oil and water which is combined with a particular antigen to boost the immune response to that antigen) in patients with progressing advanced gastric cancer was well tolerated and found to be safe (Ishikawa et al. 2014). Prostatic acid phosphatase (PAP) is a prostate-specific protein which is overexpressed in 95% of prostate tumours (Saif et al. 2014). It has been reported that PAP-114-128 (MSAMTNLAALFPPEG) prevents and reduces the growth of transgenic adenocarcinoma of mouse prostate-C1 prostate cancer cell-derived tumours (Saif et al. 2014). WT1-specific antibody and cytotoxic T lymphocyte (CTL) are generated in cancer patients which gives the idea that WT1 could be used as a vaccine for cancer. WT1 peptide vaccine (CYTWNQMNL) is under clinical trial. WT1 vaccination-related immunological responses and clinical responses, including reduction of leukaemic cells and M-protein amount in myeloma and shrinkage of solid cancer, were observed (Oka et al. 2008).

2.4.9 Venom Peptides as Cancer Drugs

Ion channels, viz. sodium(Na^+), potassium(K^+), calcium (Ca^{2+}) and chloride(Cl^-) channels, have significant role in cancer (Lang and Stournaras 2014). Modification of these voltage-gated channels may transform the cells to tumorigenic (Lang and Stournaras 2014). Literature reports suggest that venom peptides from snail, snake, scorpion, bee, etc. can potentially act as new arsenal against cancer. Venom peptides are amphipathic in nature and have multiple cationic residues (e.g. lysine, arginine, and histidine) and hydrophobic residues. They are generally strongly resistant to pH, temperature and protease degradation and exhibit selectivity to receptors (Silman et al. 2016). Such peptides execute the disruption of cell membrane either by pore formation or disaggregation of membrane lipids by micelle formation (Ma et al. 2017; Lee et al. 2008b). They are stabilized either by posttranslational modifications or have protease-resistant D-amino acid residues in the sequence and also have multiple disulphide bonds (Mahadevappa et al. 2017). Venom peptides specifically target different ion channels on the cell membrane; thereby they inhibit cancer cell proliferation (Ma et al. 2017).

Scorpion toxin can be classified on the basis of four different characteristic features, (a) involvement of ion channels (Na+, K+, Ca2+,Cl-), toxin binding specific receptor, 3-D structures of toxins and the type of induced responses such as activation or inactivation of the receptor (Quintero et al. 2013). Chlorotoxin (venom of scorpion *Leiurus quinquestriatus*) targets chloride (Cl^-) channels on a wide variety of cells (Mahadevappa et al. 2017). In vitro, chlorotoxin-conjugated cisplatin shows enhanced cytotoxicity in cervical, breast and lung cancer cells than free cisplatin (Graf et al. 2012). Combined effect of venom peptides with chemotherapeutic drugs minimizes the concentration of standard chemotherapy, decreases side effects and enhances therapeutic outcome of the chemotherapeutic drugs. Bee venom melittin is a potent inhibitor of human leukaemia cell growth and exhibits strong membrane-perturbing activity (Rady et al. 2017). Melittin readily binds to negatively charged membrane surfaces and disrupts phospholipid bilayer either by pore formation or disruption of Na^+ ion channel in particular and Ca^{2+} ion channel indirectly (van den Bogaart et al. 2008). Soricidin (toxin peptide originated from shrew) can selectively inhibit TRPV6 channels (Ca^{2+} channel) (Lo et al. 2013). Venom peptides used for cancer therapy are listed in Table 2.9.

2.4.10 Anti angiogenic Peptides

The highly proliferating cells in a malignant tumour need new blood vessel for its supply of oxygen and nutrients and removal of waste products (Nishida et al. 2006). The process of generation of new blood vessels is termed as angiogenesis. Inhibiting angiogenesis is a way for treating cancer. Rosca et al. have provided an elaborate list of anti angiogenic peptide (Rosca et al. 2011). Angiotensin inhibits angiogenesis in lung cancer (Soto-Pantoja et al. 2009). Cilengitide, a cyclized RGD pentapeptide derived from extracellular matrix, inhibits angiogenesis in glioblastoma and

Table 2.9 Source and sequences of venom peptides used as anticancer drugs

Sl no.	Venom peptide	Peptide sequence	Origin/ source	Cancer types	Targeted ion channels	Reference
1	Chlorotoxin	MCMPCFTTDH QMARKCDDCC GGKGRGKCYG PQCLCR	Scorpion	Cervical, breast and lung cancer	Cl^- ion channel	Yin et al. (2007), Graf et al. (2012)
2	Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2	Bee	Renal, lung, liver, prostate, bladder and mammary cancer	Na^+ and Ca^{2+} ion channel	Huang et al. (2013), Oršolić (2011)
3	Soricidin	DCSQD CAACS ILARP AELNT ETCIL ECEGK LSSND TEGGL CKEFL HPSKV DLPR	Shrew	Ovarian, breast, thyroid, prostate, colon cancer, leukaemia, lymphoma	Ca^{2+} ion channel	Lo et al. (2013)

refractory brain tumour in children (Reardon et al. 2008). Pentastatin-1, C16Y and C16s are also extracellular matrix-derived anti angiogenic peptides used against breast cancer (Rosca et al. 2011). P144 targets TGFβ and is anti angiogenic to skin fibrosis (Rosca et al. 2011). Chemokinostatin-1 and anginex derived from chemokines are anti angiogenic peptides and have shown effects in breast cancer and ovarian cancer, respectively (Rosca et al. 2011). A dipeptide IM862 (L-glutamine L-tryptophan) which is synthesized in the thymus gland has also exhibited anti angiogenic property (Noy et al. 2005). Sequences of anti angiogenic Peptides are listed in Table 2.10.

2.5 Role of Peptides in Epigenetics and Autophagy and as Proteasome Inhibitors

Short peptides can bind to DNA grooves, histone H1 and histone tails (Vanyushin and Khavinson 2016). These can result in change of structure of DNA double helix and hence gene expression. A few short peptides, namely, Pinealon (Glu-Asp-Arg), Pancragen (Lys-Glu-Asp-Trp), Cardiogen (Ala-Glu-Asp-Arg), Testagen (Lys-Glu-Asp-Gly) and Bronchogen (Ala-Glu-Asp-Leu), bind to DNA and presumably have epigenetic role. All these peptides were synthesized by Saint Petersburg Institute of Bioregulation and Gerontology, Russia (Vanyushin and Khavinson 2016). Bronchogen regulates the expression of MKI67, MCL1, TP53, CD79A and NOS-3 genes. Bronchogen also decreases promoter methylation in young and matured bronchoepithelial cell line (Ashapkin et al. 2015). Pancragen diminishes the level of one of the key cell differentiation genes PDX1 in young cell cultures and increases its expression in aged cultures. Pancragen also causes decrease in methylation levels at methylated sites of DNA (Ashapkin et al. 2015). It also increases the expression of NKX6-1 (required for the development of beta cells in pancreas) in younger cell cultures and NGN3 (responsible for differentiation in early stages of pancreatic beta cells) expression in aged cells (Ashapkin et al. 2015). Epigenetic effects are observed by casein-derived opioid peptides in neuroblastoma cells (Trivedi et al. 2015). Although neuropeptides facilitate malignant transformation on many types of solid tumours, recent studies show that neuropeptides can be used to regulate cancer epigenetically, mainly in the case of gastric cancer and chondrosarcoma (Galoian and Patel 2017). Romidepsin, a natural bicyclic depsipeptide isolated from bacterium *Chromobacterium violaceum*, is a FDA-approved histone deacetylase inhibitor drug, used to treat cutaneous or peripheral T-cell lymphoma (Vandermolen et al. 2011). Peptides and proteins can also regulate autophagy. Beclin, a 60 kDa protein, is an autophagy promoter, and Beclin conjugated with TAT is also a potent inducer of autophagy (Shoji-Kawata et al. 2013). Increased autophagy has shown to block HER-2-mediated breast tumorigenesis (Vega-Rubín-De-Celis et al. 2018). While promoting autophagy is beneficial for some cancer therapy, e.g. in breast cancer cell line MCF-7 (Liang et al. 1999), most of the cancer therapy involves downregulation of autophagy, viz. colon cancer, hepatic carcinoma, multiple myeloma, etc. (Thorburn et al. 2014). Autophagy inhibitory peptides derived from giant ankyrins can play a great role in cancer therapeutics (Li et al. 2018).

Table 2.10 Anti angiogenic peptide sequences

Sl no.	Peptide	Peptide sequence	Type of cancer	Reference
1	Angiotensin I	H-DRVYIHPFHL-OH	Lung	Soto-Pantoja et al. (2009), Pubchem CID: 3081372
2	Angiotensin II	H-DRVYIHPF-OH	Lung	Soto-Pantoja et al. (2009), Pubchem CID: 172198
3	Angiotensin III	H-RVYIHPF-OH	Lung	Soto-Pantoja et al. (2009), Pubchem CID: 3082042
4	Angiotensin IV	H-VYIHPF-OH	Lung	Soto-Pantoja et al. (2009), Pubchem CID: 123814
5	Cilengitide	Cyclo(-Arg-Gly-Asp-D-Phe-N-Me-Val) trifluoroacetate salt	Glioblastoma	Le Joncour and Laakkonen (2018), BACHEM Catalog Number H-8174
6	IM862	$^{L}Q^{L}W$	Kidney	Noy et al. (2005)
7	Pentastatin-1	LRRFSTMPFMFCNINNVCNF	Breast	Koskimaki et al. (2009)
8	C16Y	DFKLFAVYIKYR	Breast	Ponce et al. (2003)
9	C16S	DFKLFAVTIKYR	Breast	Ponce et al. (2003)
10	P144	TSLDASIIWAMMQN	Skin fibrosis	Medina-Echeverz et al. (2014), Santiago et al. (2005), Serrati et al. (2009)
11	Chemokinostatin-1	NGRKACLNPASPIVKKIIEKMLNS	Breast	Koskimaki et al. (2009), Karagiannis and Popel (2008)
12	Anginex	ANIKLSVQMK LFKRHIKWKI IVKLNDGREL SLD	Ovary, melanoma	Griffioen et al. (2001), Mayo et al. (2003), Mayo et al. (2001)

Inhibition of proteasome function has evolved as a very important strategy in cancer therapeutics. Bortezomib is the first proteasome inhibitor that is tested on humans and is used to treat multiple myeloma and mantle cell lymphoma (Chen et al. 2011). All the proteasome inhibitors that have undergone clinical trials are peptide based, except marizomib. Carfilzomib is an irreversible peptide epoxyketone, which is used to treat chemoresistant multiple myeloma (MM) (Ping Dou and Zonder 2014). Ixazomib is an oral proteasome inhibitor against MM (Ping Dou and Zonder 2014). Delanzomib (DLZ) shows proteasome-inhibiting activity against myeloma and lymphoma (Ping Dou and Zonder 2014). Oprozomib (OPZ) is another second-generation proteasome-based inhibitor which can be administered orally and has shown efficacy against xenograft murine model (Ping Dou and Zonder 2014). Some of the important peptides working as epigenetic modifier and proteasome inhibitors are listed in Table 2.11.

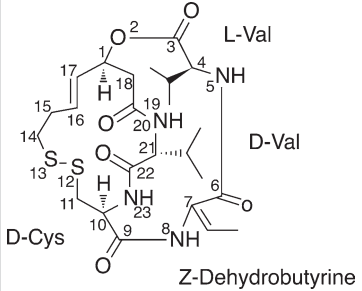
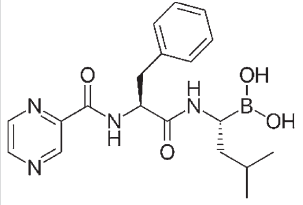
2.6 Peptide Based Combination Therapy Against Cancer

Peptide-based combination therapies are also being examined in cancer treatment. Cancer cells always devise mechanism for transformation, and if one pathway is blocked, then another alternative pathway is upregulated. Combination therapy provides a ray of hope to this problem, and it involves the use of two or more drugs with the aim to provide more therapeutic advantage than monotherapy. Combination therapy enhances the therapeutic index of the anticancer drugs by providing synergistic effects and combatting drug resistance which increases the lifespan of the cancer patients. Peptides used in combination therapy are listed in Table 2.12.

2.7 Concluding Remarks

In this chapter, we have tried to emphasize on the emerging role of peptide based therapeutics in early detection of cancer, in managing drug resistance and metastasis in cancer treatment. Additionally, we have discussed about peptide-based clinically safe molecular transporters for delivering cytotoxic drugs to cancer patients. Future development of multifunctional peptide-based therapeutics will be nature-inspired. We foresee that such bio-inspired peptides can have improved proteolytic and thermal stability and better efficacy by exploiting hybrid peptide technology. The current success story of peptide-based therapeutics demonstrates peptides as effective future therapeutics for the cancer treatment and also the treatment of unmet medical challenges.

Table 2.11 Sequences of peptides having role in epigenetics and proteasome inhibition

Sl no.	Peptide	Peptide sequence	Role	Reference
1	Pinealon	EDR	Epigenetic modifiers	Vanyushin and Khavinson (2016)
2	Pancragen	KEDW		Vanyushin and Khavinson (2016)
3	Cardiogen	AEDR		Vanyushin and Khavinson (2016)
4	Testagen	KEDG		Vanyushin and Khavinson (2016)
5	Bronchogen	AEDL		Vanyushin and Khavinson (2016)
6	Romidepsin			Vandermolen et al. (2011)
7	Bortezomib		Proteasome inhibitors	Chen et al. (2011)

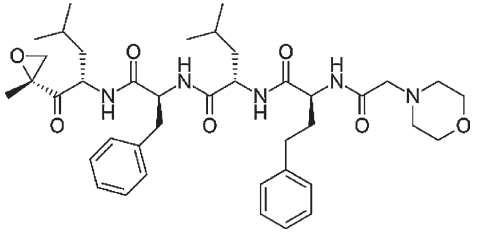
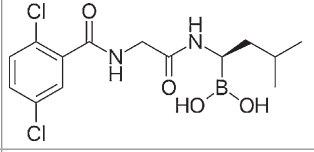
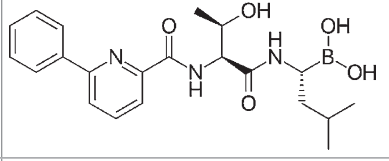
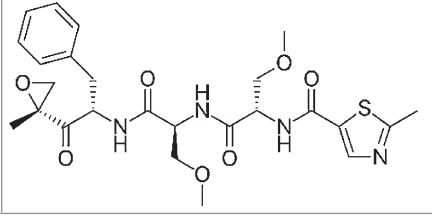
8	Carfilzomib			Ping Dou and Zonder (2014), Pubchem CID: 11556711
9	Ixazomib			Ping Dou and Zonder (2014), Pubchem CID: 25183872
10	Delanzomib			Ping Dou and Zonder (2014), Pubchem CID: 24800541
11	Oprozomib			Ping Dou and Zonder (2014), Pubchem CID:25067547

Table 2.12 Peptide sequences used in combination therapy

Sl no.	Peptide-drug combinations	Peptide sequence and conjugates	Reference
1	RGD-SSL-Dox	SSL = sterically stabilized liposomes	Xiong et al. (2005)
		Dox = doxorubicin	
2	Bac-7-ELP-p21	Bac = MRRIRPRPPRLPRPRPRPLPFPPRP	Massodi et al. (2010)
		ELP = (VPGXG)150	
		p21 = GRKRRQTSMTDFYHSKRRLIFSKRKP	
3	Bortezomib + p21-ELP-Bac	Bortezomib = proteasome-inhibiting anticancer drug	Ana-Matea et al. (2014)
4	F56-VCR-NP	F56 peptide (WHSDMEWWYLLG)	Xiao et al. (2015), Wang et al. (2014b)
		VCR = vincristine	
5	Cep55/c10orf3_193+ Cep55/c10orf3_402 + Cep55/c10orf3_283	VYVKGLLAKI + EFAITEPLVTF + LYSQRRADVQHL	Inoda et al. (2011)
6	"Peptide cocktail"	RNF43–721: NSQPVWLCL	Hazama et al. (2014)
		TOMM34–299: KLRQEVKQNL	
		KOC1(IMP-3)-508 (KTVNELQNL)	
		VEGFR1–1084: SYGVLLWEI	
		VEGFR2–169: RFVPDGNRI	
7	7-peptide cocktail vaccine	RNF43: NSQPVWLCL	Okuno et al. (2014)
		TOMM34: KLRQEVKQNL	
		FOXM1: IYTWIEDHF	
		MELK: EYCPGGNLF	
		HJURP: KWLISPVKI	
		VEGFR1: SYGVLLWEI	
		VEGFR2: RFVPDGNRI	

8	LFC131-Dox NPs	LFC 131 peptide: Tyr-Arg-Arg-Nal-Gly; Nal: 3-(2-naphthyl)-alanine	Chittasupho et al. (2014)
9	TH10-DTX-NP	TH10 peptide: TAASGVRSMH	Guan et al. (2014)
		DTX – docetaxel	
10	WT1 peptide-based vaccine + gemcitabine	WT1 peptide: CYTWNQMNL	Nishida et al. (2014)
11	KIF20A-derived peptide + gemcitabine	KVYLRVRPLL	Suzuki et al. (2014)
12	GV1001 + gemcitabine	EARPALLTSRLRFIPK	Staff et al. (2014)
13	E75 + GM-CSF	E75 = KIFGSLAFL	Clifton et al. (2016)
		GM-CSF = granulocyte-macrophage colony-stimulating factor	
14	FNIII14+ Ara C	TEATITGLEPGTEYTIYVIAL	Fukai et al. (1997), Matsunaga et al. (2008)
		Ara C = cytarabine/arabinosylcytosine	
15	D-K6L 9 + IL-12	Ac[D(K_6L_9)]-NH_2	Cichoń et al. (2014)
		IL-12 = interleukin 12	
16	LD8 + Dox	LD8 = Boc-^{L}Ala-^{D}Val-^{L}Leu-^{D}Ala-^{L}Val-^{D}Ala-^{L}Leu-^{D}Trp-OMe	Chakraborty et al. (2018)
		Dox = doxorubicin	
17	VEGFR1–1084 + S-1 + cisplatin	VEGFR1–1084 = SYGVLLWEIF	Masuzawa et al. (2012)
		S-1 = combination drug tegafur/gimeracil/oteracil	
18	VEGFR2–169 + S-1 + cisplatin	VEGFR2–169 = RFVPDGNRI	Masuzawa et al. (2012)
		S-1 = combination drug tegafur/gimeracil/oteracil	
19	BPP + cisplatin	BPP = bradykinin-potentiating peptides (EWPRPQIPP)	Wang et al. (2014e)

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Protein Palmitoylation in Cancer

3

Sonia Lobo

Abstract

Protein palmitoylation describes the posttranslational addition of the 16-carbon fatty acid, palmitate, to specific cysteine of proteins via a labile thioester bond. Unlike other forms of lipidation, such as myristoylation and prenylation, palmitoylation is reversible, allowing for dynamic regulation of protein-membrane interactions, protein trafficking between membrane compartments, protein-protein interactions, and protein function. Recent proteomic studies have revealed that the number of palmitoylated proteins in mammals is both abundant and diverse; curation of data from such studies has demonstrated that genes encoding palmitoylated proteins constitute 10% of the genome and that palmitoylation is enriched equally in cancer, at neuronal synapses, and in disorders of the nervous system. This suggests that a disruption in the homeostatic balance of protein palmitoylation can have critical pathophysiological consequences. Palmitoyl acyltransferases (PATs) catalyze the transfer of palmitate to a substrate, while depalmitoylation is mediated by acyl-protein thioesterases (APTs). To date, nearly half of the 23 genes that encode PATs and several APT genes have been linked to tumorigenesis, representing important targets for cancer drug development. While convincing evidence for a role for aberrant palmitoylation in cancer remains to be established, the number of cancer-related signaling proteins and networks in which palmitoylation plays a pivotal role is large and growing. Developing pharmacological modulators of palmitoylation to prevent or reverse cancer progression will require that they be developed within the context of well-characterized PAT-/APT-related signaling systems implicated in cancer.

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Palmitoylation · Palmitoyl acyltransferases · Acyl-protein thioesterases · Posttranslational modification · Cancer

3.1 Introduction

Proteins can be modified by four types of lipids—attachment of myristate or palmitate represents forms of acylation, while the addition of farnesyl or geranylgeranyl groups represents forms of prenylation (Fig. 3.1). Palmitoylation describes the enzymatic attachment of the 16-carbon fatty acid, palmitate, or other long-chain fatty acids to specific cysteine residues of a protein through a thioester bond. Two types of palmitoylation exist—*S*-palmitoylation in which palmitate is added to the thiol side chain of a cysteine residue via a labile thioester bond and *N*-palmitoylation in which palmitate is attached to an N-terminal cysteine via a stable amide bond (Linder and Deschenes 2007). *S*-palmitoylation occurs posttranslationally and is unique among other forms of lipidation, such as myristoylation and prenylation, due

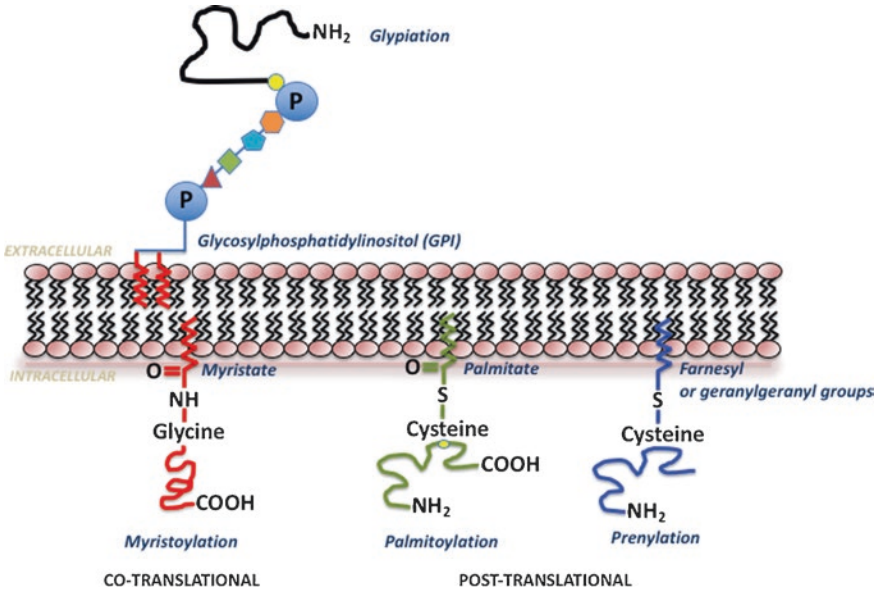


Fig. 3.1 Protein modification by lipids. Proteins can be modified by four types of lipids: myristate or palmitate represents forms of acylation, while the addition of farnesyl or geranylgeranyl groups represents forms of prenylation. Each type of modification targets proteins (to which they are attached) to unique subcellular locales. This ability is likely due to their varying chain length, degree of saturation, and their physical position on the proteins. Myristoylation occurs co-translationally via a stable amide bond to the N-terminal glycine of a protein, while farnesyl and geranylgeranyl are C-term thioethers—all stable covalent bonds

to the reversibility of the thioester bond, allowing for dynamic regulation of protein-membrane interactions, protein trafficking between membrane compartments, protein-protein interactions, and protein function. Different families of palmitoyl acyltransferases (PATs) regulate the two forms of palmitoylation—*S*-palmitoylation by a family of multipass transmembrane (TM) proteins called DHHC proteins (Mitchell et al. 2006) and *N*-palmitoylation by a family of multipass TM proteins termed membrane-bound O-acyltransferases (MBOAT) (Hofmann 2000). PATs have been shown to palmitoylate both TM and peripheral membrane proteins, including many G protein-coupled receptors (GPCRs), viral envelope proteins, ion channels, and ionotropic neurotransmitter receptors (Kang et al. 2008a). Peripheral membrane proteins become stabilized following *S*-palmitoylation, and many palmitoylated signaling proteins are dual lipidated with a prenyl or N-myristoyl group followed by palmitate (Fig. 3.2) (Shahinian and Silvius 1995). Most MBOAT proteins do not function as PATs but instead catalyze transfer of long-chain fatty acids to hydroxyl groups of other lipophilic molecules.

The ability to identify PAT/substrate pairs and to annotate palmitoylated proteins on a larger scale has been improved by methodological advances in higher-throughput proteomic quantification and site-specific labeling. Recent proteomic studies have revealed that the number of palmitoylated proteins in mammals is diverse and more abundant than previously thought (Martin et al. 2012; Yang et al. 2010; Sanders et al. 2015); curation of data from such studies has demonstrated that 1838 genes encode palmitoylated proteins (10% of the genome) and that palmitoylation is enriched equally in cancer, at neuronal synapses, and in disorders of the nervous system (Sanders et al. 2015). This suggests that a disruption in the homeostatic balance of protein palmitoylation can have critical pathophysiological consequences.

In this chapter, we will focus on the emerging relationship between *S*-palmitoylation and cancer, including the role that palmitoylated proteins play in tumorigenesis, proliferation, and metastasis, as well as their potential involvement

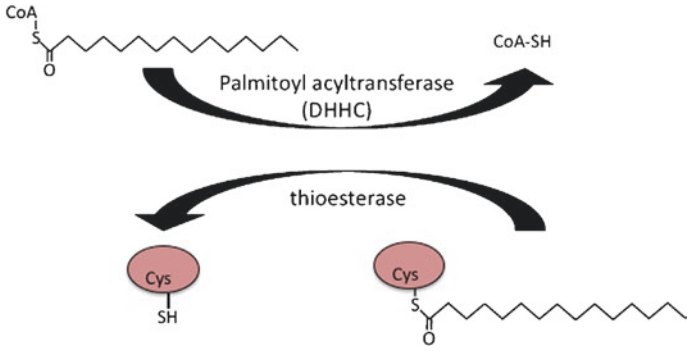


Fig. 3.2 Illustration depicting protein *S*-palmitoylation and depalmitoylation reactions. Proteins can be transiently modified with palmitate, which is added to a protein by a palmitoyl acyltransferase (PAT) and removed by an acyl-protein thioesterase (APT)

as therapeutic targets. While convincing evidence for a role for aberrant palmitoylation in cancer remains to be established, there are a growing number of cancer signaling networks in which palmitoylation plays a key role. In many cases, palmitoylation is a key determinant for the oncogenic or tumor-suppressive properties of a protein. Further, nearly half of the genes encoding PATs and several APTs have been implicated in tumorigenesis and metastasis. Identifying all palmitoylated proteins and the PATs/APTs that palmitoylate/depalmitoylate them is one of the most important fundamental questions facing this rapidly emerging field. Such knowledge will help to elucidate the potential ramifications of malfunctioning PATs/APTs on downstream signaling events, relating clinical manifestations of cancer with specific PAT/APT mutations and creating opportunity for identification of new therapeutic targets.

3.2 Enzymatic Mechanisms of *S*-Palmitoylation

It is now well established that palmitoylation is mostly enzymatic and regulated by two families of enzymes—the DHHC domain containing PATs which mediate the addition of palmitate to target substrates and the APTs which remove palmitate (Fig. 3.3) (Conibear and Davis 2010). The highly conserved Asp-His-His-Cys cysteine-rich domain (DHHC-CRD) not only defines PATs but also is directly involved in the transfer of palmitate to a substrate (Fig. 3.4a). These enzymes use a two-step kinetic mechanism involving autoacylation of the DHHC motif cysteine and subsequent acyl transfer to the substrate protein (Mitchell et al. 2006; Jennings and Linder 2012). Notably, palmitoylation can occur in cascades, with one PAT controlling the activity of another, as was recently demonstrated for DHHC16 and DHHC6 (Abrami et al. 2017). Until recently, depalmitoylation of cytosolic cysteine residues has been attributed to APT1/LYPLA1 and two related enzymes APT2/LYPLA2 and LYPLAL1 (APTL1). Compared to PATs, far less is known about the mechanisms that mediate protein deacylation. These are cytoplasmic enzymes, but they also localize on membranes by modification with palmitate. Since APT and APT2 are both palmitoylated, autoregulatory mechanisms are proposed where the depalmitoylating enzymes control physical access to their substrates on cell membranes (Kong et al. 2013; Vartak et al. 2014).

In humans, there are at least 23 members of the *ZDHHC* PAT gene family with possible or at least partially overlapping mechanisms/motifs for substrate recognition, specificity, and activity. As predicted by hydropathy analyses, the DHHC proteins encoded by these genes all contain four or more TM domains (Fig. 3.4a). Predictions using TopPred II 1.1 as presented by Ohno et al. show that most PATs have an even number of TM domains with the catalytic DHHC-CRD domain facing the cytosol and usually located between the second and third TM domain (Fig. 3.4b) (Ohno et al. 2006). In addition to the importance of PAT membrane topology, their membrane system of residence is likely to be an important aspect of their function. Most mammalian DHHC proteins have been shown to localize to the ER and Golgi (Ohno et al. 2006); however, some have also been localized to the PM or to

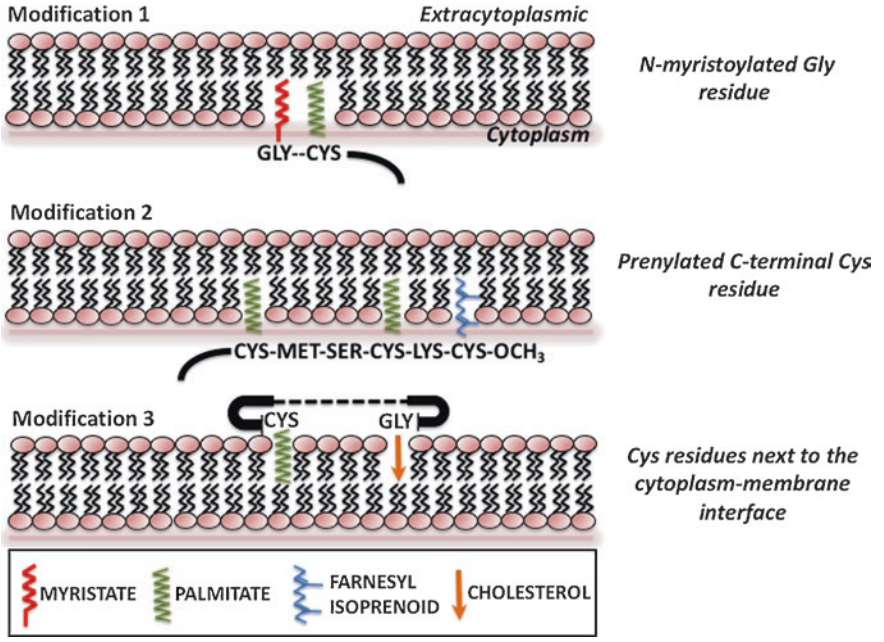


Fig. 3.3 Predictors of protein palmitoylation. The search for PAT-substrate specificities has been hindered in part by the fact that there are many proteins for which the palmitoylated cysteine residues are not associated with a defined consensus sequence. The exceptions are cysteine residues that are near an N-myristoylated glycine residue or a prenylated C-terminal cysteine residue or cysteine residues that are next to the cytoplasm-membrane face

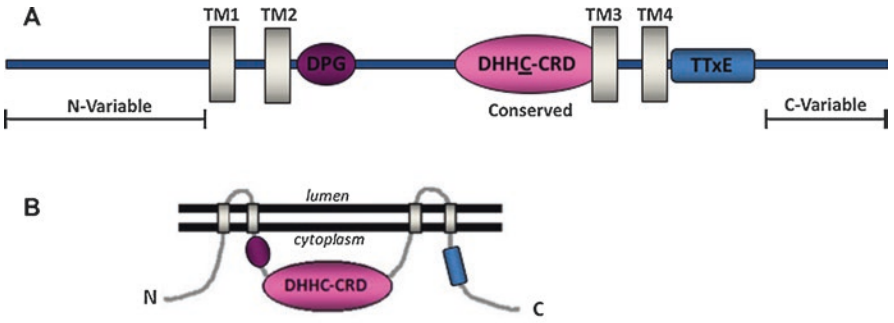


Fig. 3.4 Structure and membrane topology of palmitoyl acyltransferases (PATs). (**a**) In humans there are 23 members of the PAT gene family. PATs contain a highly conserved Asp-His-His-Cys (DHHC) cysteine-rich domain (CRD) motif that is directly involved in the transfer of palmitate to a substrate. They have the greatest diversity at the amino acid level in the N- and C-terminal, cytoplasmic tails, and most also have a conserved DPG (aspartate-proline-glycine) motif and TTxE (Thr-Thr-Xxx-Glu) motif, but their role in PAT function is not yet known. (**b**) All PATs contain four or more transmembrane (TM) domains as predicted by hydropathy analyses. Most have an even number of TM domains with the DHHC-CRD motif and the N- and C-termini in the cytoplasm and typically located between the second and third TM domain

endocytic vesicles (Ohno et al. 2006; Greaves and Chamberlain 2011; Planey et al. 2009). Yet, little is known about how these proteins achieve their respective localizations. Knockdown studies of individual DHHC enzymes have demonstrated that for certain substrates, efficient *S*-palmitoylation requires individual enzymes (Roth et al. 2006; Zhang et al. 2008). Thus, the relative expression of ZDHHC genes in vivo is likely important in determining substrate specificity in different cell types.

As is the case for PATs, the substrate selectivity of depalmitoylating enzymes remains to be defined, but similar factors such as expression level and cell context likely affect their substrate specificity. The APT family includes APT1, APT2, APTL1, and PPT1 and PPT2 (protein palmitoyl thioesterase). PPT1 and PPT2 catalyze the deacylation of protein substrates in the lysosome (Verkruyse and Hofmann 1996), whereas APT1 and APT2 are cytosolic enzymes encoded by *LYPLA1* and *LYPLA2*, respectively. APT1 and APT2 were originally isolated as lysophospholipases and later demonstrated to be effective as cytosolic protein thioesterases (Sugimoto et al. 1996; Tomatis et al. 2010; Toyoda et al. 1999; Zeidman et al. 2009). APT1 has been shown to regulate the acylation-deacylation cycle of several cytoplasmic proteins including Gα subunits, H-Ras, endothelial nitric oxide synthase, and SNAP-23 (Kong et al. 2013; Dekker et al. 2010; Yeh et al. 1999). APT1 also catalyzes its own depalmitoylation and that of APT2 promoting dynamic palmitoylation of both thioesterases (Vartak et al. 2014). APT2 is 68% homologous to APT1 (Toyoda et al. 1999) but catalyzes the depalmitoylation of semisynthetic N-Ras more efficiently than APT1 (Rusch et al. 2011), and specifically depamitoylates the axonal, peripheral membrane-associated GAP-43 (Tomatis et al. 2010) and hydrolyzes prostaglandin glycerol esters (Manna et al. 2014). Both APT1 and APT2 undergo palmitoylation on Cys-2, and blocking palmitoylation adversely affects their membrane localization and that of their substrates. APTL1 (*LYPLAL1*) is a distant homolog of APT1 that was shown to have limited function as a palmitoyl thioesterase due to its narrow substrate-binding pocket and preference for short-chain lipid substrates in vitro (Burger et al. 2012); however, genetic studies show that it is upregulated in obesity (Fox and Gartenberg 2012).

The disproportionately small number of APTs relative to PATs is reminiscent of the disparity between phosphatases (<100) and protein kinases (>500) in human cells, suggesting that APTs may function as broad specificity enzymes that depalmitoylate a wide range of substrates, while PATs harbor the specificity for dynamic palmitoylation. Alternatively, this disparity may reflect the fact that only a limited subset of proteins undergo palmitoylation turnover. Yet, it is also possible that many more thioesterases exist but have yet to be identified. Indeed, recent studies have discovered the membrane-anchored alpha/beta-hydrolase domain-containing protein 17 (ABHD17) proteins as depalmitoylating enzymes that regulate N-Ras and PSD-95 palmitoylation cycles in mammalian cells (Lin and Conibear 2015; Yokoi et al. 2016). ABHD12 and ABHD13 also exhibited depalmitoylating activities, albeit weaker than ABHD17 when tested against PSD-95 as the substrate (Yokoi et al. 2016). These findings suggest that the family of depalmitoylating enzymes may be substantially broader than previously thought (for a comprehensive review, see Won et al. (2018)).

3.3 PATs, APTs, and Cancer

The increasing body of evidence from genetic studies and animal model systems has demonstrated the involvement of PAT-/APT-mediated *S*-palmitoylation in numerous and diverse pathological conditions including neurological/neuropsychiatric disorders, dermatological pathologies, diabetes, osteoporosis, and cancer. The significant association of *S*-palmitoylation with both nervous system diseases and cancers suggests that disrupting the homeostatic balance of protein palmitoylation may lead to cell death or uncontrolled cell growth depending on the proteins involved (Sanders et al. 2015). To date, nearly half of the genes encoding PATs and several APTs have been linked, in varying degrees, to cancer. These include the PATs *ZDHHC2*, *ZDHHC3*, *ZDHHC5*, *ZDHHC7*, *ZDHHC9*, *ZDHHC11*, *ZDHHC13*, *ZDHHC14*, *ZDHHC17*, *ZDHHC20*, and *ZDHHC21* and the APTs *LYPLA1* and *LYPLA2*. Altered expression of individual PAT/APT enzymes has been shown to alter cancer-related signaling in a tumor suppressor or oncogenic manner depending on the cellular context (Fig. 3.5). It is clear from the examples described below that aberrant *S*-palmitoylation can have significant and deleterious effects on cancer-related signaling networks (for a comprehensive review, see Yeste-Velasco et al. (2015)).

3.3.1 ZDHHC2

One compelling example linking perturbation of palmitoylation cycling to cancer involves *ZDHHC2*. Deletion or epigenomic silencing of *ZDHHC2* (previously known as *REAM*, for reduced expression associated with metastasis (Oyama et al. 2000)) has been detected in various metastatic cancers including breast (Anbazhagan

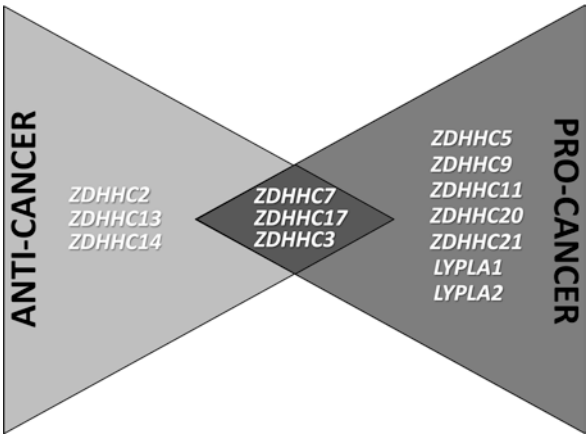


Fig. 3.5 Venn diagram illustrating the potential role of PAT and APT family members in human cancer. The overlapping region includes those PATs for which both anticancer and pro-cancer roles have been described

et al. 1998; Yaremko et al. 1996), lung (Fujiwara et al. 1994; Ohata et al. 1993), urinary bladder (Knowles et al. 1993), prostate (Bova et al. 1993), colorectal (Oyama et al. 2000; Fujiwara et al. 1993a), and hepatocellular cancers (Emi et al. 1993; Fujiwara et al. 1993b), suggesting that palmitoylation of *ZDHHC2* substrates regulates metastatic potential. Further, data from cBioPortal analysis reveal extensive evidence for *ZDHHC2* gene deletions particularly in breast, lung, and prostate tumors (Resh 2017). DHHC2 has been shown to regulate the tetraspanins CD9 and CD151 (Sharma et al. 2008) and CKAP4 (Planey et al. 2009; Zhang et al. 2008), which may provide new mechanistic insights into metastatic disease.

CKAP4 is a reversibly palmitoylated, type II TM protein that has been shown to anchor rough ER to microtubules in epithelial cells. More recently, CKAP4 has been identified as a functional receptor for antiproliferative factor (APF)—a sialoglycopeptide secreted from bladder epithelial cells in patients with interstitial cystitis (IC) (Conrads et al. 2006; Chavda et al. 2017). Importantly, silencing *ZDHHC2* expression with siRNA abolishes the antiproliferative effects of APF in urologic and non-urologic cancer cell lines (Planey et al. 2009). *S*-Palmitoylation of CKAP4 by DHHC2 is required for it to exit the ER and move to the PM; without CKAP4 surface expression, APF is unable to inhibit cellular proliferation or alter the expression of genes involved in cancer progression (Matika et al. 2012; Zacharias et al. 2012) and IC (reviewed in Keay (2008)). More recently, expression of CKAP4 and DHHC2 expression were shown to correlate with disease progression and metastasis in patients with hepatocellular carcinoma (HCC) (Li et al. 2014; Peng et al. 2014). Patients with high CKAP4 expression had a favorable overall survival and a longer disease-free survival compared with those with low expression. In 87.6% of these cases, microarray analysis by immunohistochemistry revealed low *ZDHHC2* expression (Li et al. 2014). Similarly, reduced *ZDHHC2* expression was associated with lymph node metastasis and independently predicted an unfavorable prognosis in gastric adenocarcinoma patients (Yan et al. 2013). These findings suggest that *ZDHHC2* may provide prognostic and therapeutic value in HCC and other cancers.

The tetraspanin proteins, CD9 and CD151, have also been identified as substrates of DHHC2. Tetraspanins are molecular scaffolds that distribute proteins into highly organized microdomains consisting of adhesion, signaling, and adaptor proteins. Consequently, these membrane proteins can act as both metastasis suppressors and promoters, contributing to cell adhesion, motility, invasion, activation, and proliferation (Termini and Gillette 2017). CD9 is itself a suspected tumor suppressor (Ikeyama et al. 1993; Miyake et al. 2000), while studies suggest that CD151 promotes metastasis by activating α3β1 integrin-dependent tumor cell adhesion and migration (Kumari et al. 2015). Suppression of DHHC2-mediated palmitoylation of CD9 in A431 cells causes them to undergo what appears to be epithelial-mesenchymal transition (EMT), shifting to a dispersed state with cell-cell contact decreased. DHHC2 palmitoylation promoted physical associations between CD9 and CD151 and between alpha 3 integrin and other proteins and also protected CD151 and CD9 from lysosomal degradation (Sharma et al. 2008).

3.3.2 ZDHHC3

ZDHHC3 encodes the Golgi-resident PAT, DHHC3 (GODZ), whose elevated expression has been linked to malignant and metastatic breast cancer as well as to colon and prostate cancers (Sharma et al. 2017; Sharma and Hemler 2017). In a recent study, *ZDHHC3* upregulation correlated with significantly diminished overall survival of patients with breast cancer; further, a marked decrease in the sizes of both primary tumors and metastatic colonies was observed in human MDA-MB-231 xenograft models following *ZDHHC3* ablation. The authors concluded that DHHC3-mediated protein palmitoylation supports breast tumor growth by modulating cellular oxidative stress and senescence (Sharma et al. 2017). This pro-tumor role for DHHC3 is also supported by its role in regulating the function, expression, and stability of lamin-binding integrin α6β4. α6β4 plays a key role during tumor cell progression, metastasis, and angiogenesis and is involved in cell motility and invasion through expression of Src. *ZDHHC3* knockdown diminished integrin-dependent signaling through Src, cellular cable formation, and beta 4 phosphorylation on key amino acids, indicating that it is a substrate for DHHC3 (Sharma et al. 2012). Interestingly, levels of integrin β4 (ITG β4) palmitoylation have been shown to correlate with the invasive potential of breast cancer cells, and curcumin treatment prevented ITG β4 palmitoylation and thus its lipid raft localization and signaling activity by blocking autoacylation of DHHC3 (Coleman et al. 2015). Collectively, these data suggest that DHHC3 may be a good therapeutic target for cancer.

In contrast to the studies described above, *ZDHHC3* has also been shown to have anticancer-promoting activities in squamous cell cervical carcinoma and a pro-survival effect on patients with breast cancer. Loss of *ZDHHC3* was shown to be associated with squamous cell cervical carcinoma through the downstream effects of the DHHC3 substrate DR4 or TRAIL-R1 (Choi et al. 2007; Oh et al. 2012). Palmitoylation of DR4 by DHHC3 localizes it to the PM where it can bind tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) to induce apoptosis. *ZDHHC3* expression is highly downregulated in human HCC cells that show resistance to TRAIL, but reconstitution of *ZDHHC3* expression enhances the targeting of DR4 to the cell surface and restores TRAIL sensitivity (Oh et al. 2012). The *ZDHHC3* gene is located in the cytogenetic band 3p21.31, which has been linked to early invasive breast cancer (Killary et al. 1992; Rowley et al. 1996). Several studies indicated the presence of a tumor suppressor locus within this region and established links to clinically early-stage sporadic breast tumors (Martinez et al. 2001; Sekido et al. 1998; Todd et al. 1996). Recently, the presence of an 11-base pair microsatellite allele embedded in the *ZDHHC3* 3' UTR was shown to be statistically significant in breast cancer samples from African American women. *ZDHHC3* messenger RNA expression was also lower in African American women than in white patients with cancer; however, the presence of any alterations in *ZDHHC3* was linked to breast cancer mortality in both African Americans and whites (Kinney et al. 2017). The mean overall survival for patients without *ZDHHC3* alterations versus those with alterations was more than twofold higher; hence, *ZDHHC3* may be an important biomarker for breast cancer survival (Kinney et al. 2017).

3.3.3 ZDHHC5

The *ZDHHC5* gene is located in a region of chromosome 11q12.1 that is associated with a high rate of chromosomal translocation, variation, and cancer development (Abe et al. 2016; Vijai et al. 2013). *ZDHHC5* has been shown to have oncogenic capacity in non-small cell lung cancer (NSCLC) cell lines where it is highly expressed and stimulates the proliferation and anchorage-dependent and anchorage-independent colony formation. siRNAs directed against *ZDHHC5* inhibited the growth of multiple NSCLC lines but not normal human bronchial epithelial cell (HBEC) lines; further, *ZDHHC5* silencing markedly reduced in vitro cell proliferation, colony formation, and cell invasion, while transfection of a wild-type DHHC5 plasmid (but not a catalytically inactive DHHC5) restored these phenotypes. Tumor xenograft formation was also severely inhibited by *ZDHHC5* knockdown and rescued by *ZDHHC5* expression demonstrating its oncogenic capacity in NSCLC.

ZDHHC5 overexpression has also been noted in glioma compared with normal brain tissue (Chen et al. 2017a). Glioma patients with mutations in p53 (> 30% of cases) show therapeutic resistance and poor outcomes. Chen et al. demonstrated that mutant p53 transcriptionally upregulated *ZDHHC5* and the nuclear transcription factor NF-Y. The physical interaction between the mutant p53 and NF-Y resulted in aberrant upregulation of *ZDHHC5*, which contains four potential NF-Y binding sites in its promoter. Importantly, *ZDHHC5* mRNA and protein levels increased with glioma grade, and *ZDHHC5* was shown to contribute to the development of glioma by promoting the self-renewal and tumorigenicity of glioma stem cell cultures by altering the palmitoylation and phosphorylation status of the histone methyltransferase and tumor suppressor, EZH2 (Chen et al. 2017a). Thus, *ZDHHC5* represents a potential target for p53-mutant gliomas.

3.3.4 ZDHHC7

ZDHHC7 is one of several putative tumor suppressor genes (TSGs) residing on whole-arm chromosome 16q, which is often lost in breast cancers (Hungermann et al. 2011). According to cBioPortal analysis, *ZDHHC7* is also deleted in 10% of prostate adenocarcinoma, ovarian, and breast cancer cases (Resh 2017). Recently, palmitoylation of the estrogen, progesterone, and androgen steroid hormone receptors by DHHC7 and DHHC21 has been shown to be critical for their PM localization and function in mediating rapid tissue-specific responses to steroid hormones (Acconcia et al. 2005; Pedram et al. 2012). For example, estrogen receptor-α (ERα) mediates fast cell responses to 17β-estradiol (E2) through the activation of signaling cascades such as the MAPK/ERK and PI3K/AKT murine thymoma viral oncogene homolog 1 pathways. These rapid effects require a population of the ERα located at the cell PM through physical association with caveolin-1 and promoted by palmitoylation (Pedram et al. 2007). La Rosa et al. recently reported that the lack of palmitoylation renders ERα more susceptible to E2-dependent degradation, blocks ERα S118 phosphorylation, and prevents E2-induced ERα estrogen-responsive

element (ERE)-containing promoter occupancy (La Rosa et al. 2012). As a result, ERα transcriptional activity is prevented and the receptor directed to the nuclear matrix subnuclear compartment. Thus, ERα palmitoylation appears to be required for the initial events of E2-induced activation of ERα transcriptional activity (e.g., cofactors and ERα promoter recruitment) (La Rosa et al. 2012).

ZDHHC7 has also been shown to have an anticancer function through palmitoylation of the tumor suppressor Fas (CD95)—a death receptor belonging to the TNF receptor superfamily. Fas palmitoylation occurs on Cys-199 and is critical for its ability to trigger cell death in colorectal cancer (CRC) cells (Rossin et al. 2015). Silencing or overexpressing *ZDHHC7*, respectively, reduces or enhances Fas palmitoylation, and DHHC7 co-immunoprecipitates with Fas. DHHC7-mediated palmitoylation of Fas also regulates its stability and prevents its degradation through lysosomal proteolysis, thereby ensuring proper Fas expression at the cell surface (Rossin et al. 2015).

Recently, Chen and colleagues showed that DHHC7 regulates the tumor suppressor function of Scribble (Scrib)—a protein essential for the maintenance of epithelial cell polarity. *S*-Palmitoylation of two membrane-proximal cysteines by DHHC7 regulates Scrib's ability to suppress oncogenic signaling pathways, including MAPK, PI3K/AKT, and YAP (Chen et al. 2016). Palmitoylation-deficient mutants of Scrib and knockout of *ZDHHC7* led to Scrib mislocalization, YAP activation, and disruption of Scrib's suppressive activities in H-Ras(V12)-induced cell invasion.

In contrast to the examples above, *ZDHHC7* may promote tumorigenesis through palmitoylation of its substrate, neural cell adhesion molecule (NCAM). NCAM facilitates ovarian epithelial carcinoma cell migration and invasion in vitro and promotes metastasis in mice through its interactions with the fibroblast growth factor (FGF) receptor (Zecchini and Cavallaro 2010). Activation of FGF receptor(s) by FGF2 leads to palmitoylation of the two NCAM isoforms, NCAM140 and NCAM180, translocation of NCAM to GM1 ganglioside-containing lipid rafts, and stimulation of neurite outgrowth of hippocampal neurons. Importantly, FGF2-stimulated neurite outgrowth was inhibited by mutation of the NCAM140 or NCAM180 palmitoylation sites. DHHC7 most strongly stimulated palmitoylation of NCAM, and enzyme activity was enhanced by FGF2 (Ponimaskin et al. 2008).

3.3.5 ZDHHC9, ZDHHC11, ZDHHC17, and ZDHHC20

Several PATs including *ZDHHC9*, *ZDHHC11*, *ZDHHC17*, and *ZDHHC20* have been shown to promote cancer-related signaling as a result of their overexpression. *ZDHHC9* is generally considered to be oncogenic, as it is strongly upregulated in some adenocarcinomas of the gastrointestinal tract at the transcript and protein levels (Mansilla et al. 2007). Overexpressing *ZDHHC9* promotes Ras PM localization and transformation of mammalian cells, while repressing it diminishes it, suggesting that DHHC9 selectively palmitoylates Ras proteins in the trans-Golgi compartment to facilitate sorting to the PM via the trans-Golgi network—a role that is most

certainly critical for Ras-driven cancers (Young et al. 2014). *ZDHHC11* displays characteristics that strongly suggest it is oncogenic when overexpressed. *ZDHHC11* is present on a region of chromosome 5 (5p15.33) that has a high incidence of additional genomic copies in bladder cancer cases of high-grade and advanced-stage disease and progression (Yamamoto et al. 2007) and in NSCLC (Kang et al. 2008b).

Overexpression of *ZDHHC17* has the ability to induce colony formation and anchorage-independent growth in cell culture and tumors in mice, and DHHC17 has been shown to palmitoylate oncogenic RAS proteins (Ducker et al. 2004). Furthermore, *ZDHHC17* expression is upregulated in 70% of patients with colon, stomach, and breast tumors; 60% of lung tumors; and 50% of prostate tumors and increased in stage II and III tumors of the breast, colon, and stomach (Ducker et al. 2006).

Overexpression of *ZDHHC20* has also been demonstrated in several types of human tumors, including breast, ovarian, and prostate (Draper and Smith 2010). In NIH3T3 cells, *ZDHHC20* upregulation caused phenotypic changes consistent with cellular transformation, including colony formation in soft agar, decreased contact inhibition of growth, and increased proliferation under low serum conditions. More recently, Jung and colleagues analyzed the triple-negative breast neoplasm (TNBN) gene network using gene expression data from The Cancer Genome Atlas. They identified *ZDHHC20* as one of two oncogenes in the TNBN gene regulatory network; however, they did not find meaningful fold changes in *ZDHHC20* expression between the triple-positive breast neoplasm (TPBN) and TNBN gene expression data (Jung et al. 2017). This finding along with recent data demonstrating that inactivation of DHHC20 increased cancer cell responsiveness to epidermal growth factor receptor (EGFR) inhibitor therapy suggests that targeting DHHC20 may be an effective clinical approach to treat TNBCs that are inherently resistant to EGFR targeted therapy (Bollu et al. 2015; Kharbanda et al. 2017; Runkle et al. 2016).

3.3.6 ZDHHC13

Perez et al. have described a protective role for *ZDHHC13* in skin carcinogenesis (Perez et al. 2015). They identified a nonsense base mutation in *ZDHHC13* (Zdhhc13luc) that generates a truncated DHHC13 protein, with no PAT activity. Mice homozygous for this mutation developed abnormalities in hair and skin homeostasis and had increased epidermal cell proliferation compared to wild-type skin following short-term treatment with 12-O-tetradecanoyl-phorbol-13-acetate and acute UVB exposure. *Zdhhc13luc/Zdhhc13luc* mice also displayed significantly higher tumor multiplicity and malignant progression of papillomas relative to their wild-type littermates (Perez et al. 2015).

DHHC13 has also been shown to interact with and palmitoylate the melanocortin-1 receptor (MC1R) activating its signaling (Chen et al. 2017b). MC1R activation triggers increased pigmentation, ultraviolet-B-induced G1-like cell cycle arrest, and control of senescence and melanomagenesis in vitro and in vivo. DHHC13-mediated palmitoylation of MC1R protected against melanoma, indicating that *ZDHHC13* upregulation or inhibition of depalmitoylation might have therapeutic value in

preventing melanomagenesis in individuals with red hair color variants. Notably, a novel sequence motif termed zDABM has been identified in a number of proteins recognized by the ankyrin repeat (AR) domain of DHHC13 and DHHC17 (Lemonidis et al. 2015, 2017). Several of these proteins are involved in cytoskeletal organization, cell communication, and regulation of cell signaling, including Sprouty and SPRED which are known negative regulators of MAPK signaling and potential tumor suppressors; hence, further evaluation and functional studies of these potential substrates may reveal how *ZDHHC13* protects against skin carcinogenesis and melanomagenesis and how *ZDHHC17* can promote tumorigenesis (Lemonidis et al. 2017).

3.3.7 ZDHHC14

ZDHHC14 expression has been linked to several types of cancer with both pro- and anticancer activities. Several reports have demonstrated that *ZDHHC14* upregulation is associated with aggressiveness and metastasis in lymph node-positive tongue squamous cell carcinoma (TSCC) (Onda et al. 2009) and gastric cancer (Anami et al. 2010). In scirrhous-type gastric cancer, which is highly aggressive and has a poorer prognosis, *ZDHHC14* overexpression is associated significantly with depth of tumor invasion, undifferentiated histology, and scirrhous pattern (Oo et al. 2014). In patients with acute biphenotypic leukemia, activation of *ZDHHC14* occurs through the chromosomal translocation t(6:14)(q25;q32). *ZDHHC14* overexpression inhibits leukocyte differentiation and is observed in biphenotypic leukemia, in subsets of acute myeloid leukemia with unfavorable prognosis, and in leukemia progenitor cells indicating a potential role in leukemia (Yu et al. 2011).

ZDHHC14 has also recently been identified as a novel TSG that is commonly downregulated in testicular germ cell tumors and prostate cancer, suggesting that it has an anticancer function. Single nucleotide polymorphism array analysis identified a frequent small deletion of 6q25.3 affecting the *ZDHHC14* gene in testicular germ cell tumors (Yeste-Velasco et al. 2014). Not only was *ZDHHC14* expression reduced at the RNA and protein levels in testicular tumors but also in prostate cancer clinical samples and cell lines. Further, inducible *ZDHHC14* upregulation reduced cell viability and increased caspase-dependent apoptosis. These in vitro findings were confirmed in a mouse xenograft model, demonstrating that *ZDHHC14* inhibits tumorigenesis.

3.3.8 ZDHHC21

As mentioned previously, *S*-palmitoylation localizes a fraction of ERα to the PM of cells where it interacts with caveolin-1 and initiates signaling pathways leading to cell proliferation (i.e., ERK and PI3K/AKT activation) (Pedram et al. 2012). As mentioned previously, DHHC7 and DHHC21 are critical for ERα palmitoylation and rapid membrane-initiated E2 signaling, as disrupting the expression of either

enzyme eliminates PM ER localization and E2 proliferative effects (Acconcia et al. 2005). Changes in *ZDHHC21* expression and/or activity may contribute to signaling outcomes observed in some breast cancers such as kinase activation and enhanced extranuclear ER localization. Indeed, dysregulation of *ZDHHC21* gene has been reported in the Oncomine database with significantly higher expression levels in human breast cancer versus normal breast tissue (Pedram et al. 2012). Hence, targeting *DHHC21* could serve as an effective approach to antagonize estrogen proliferative effects.

A single amino acid deletion in DHHC21, resulting in loss of PAT activity, has recently been mapped to the depilated mutant mouse model (Mill et al. 2009); however, it is not clear if this phenotype can be attributed to aberrant steroid hormone signaling. A detailed study of the phenotype demonstrates that lack of DHHC21 palmitoylation results in hyperplasia of the interfollicular epidermis and sebaceous glands and delayed differentiation of the hair shaft (Mill et al. 2009). While the exact DHHC21 substrate(s) responsible for the phenotype have not been identified, Fyn, a member of the Src family of protein tyrosine kinases, was identified as a target and was mislocalized in the mutant mice hair follicles (Mill et al. 2009). Src family kinases (SFKs) like Src and Fyn (as well as Lck, Lyn, Yes, and Hck) participate widely in signaling pathways that regulate tumorigenesis, and palmitoylation is necessary for their association with the PM and their ability to transduce signals. A recent study showed that Src, Fyn, and Lyn exhibit ranked tumorigenic potential that is regulated in part by posttranslational palmitoylation (Cai et al. 2011). Hence, increased palmitoylation of SFKs by overexpression of PATs like *DHHC21* could promote tumorigenesis and cancer progression.

ZDHHC21 has recently been identified as part of a seven-gene signature that predicts overall survival of patients with CRC (Chen et al. 2017c). Chen et al. analyzed CRC tissues from 64 patients using gene arrays. Univariate survival analysis and subsequent KEGG analysis revealed a cohort of genes involved in pathways such as endocytosis, axon guidance, spliceosome, ubiquitin-mediated proteolysis, and Wnt signaling. A likelihood-based survival modeling approach further revealed a seven-gene signature that included *CCBLI*, *NHLRC3*, *PNPO*, *PRR14L*, *PPIP5K2*, *PTPRB*, and *ZDHHC21*. All seven genes in this signature, including *ZDHHC21*, were differentially expressed in CRC tissues as compared with adjacent normal tissues when two independent data sets were examined. Notably, *ZDHHC21* expression was upregulated in CRC, indicating a potential role in CRC initiation and progression (Chen et al. 2017c).

In contrast to the potential cancer-promoting functions described above, *DHHC21* has been shown to regulate platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31)—a cell adhesion and signaling molecule that potently suppresses apoptosis in a variety of cellular systems. PECAM-1 expression has been linked to a variety of human cancers, particularly vascular and hematopoietic cancers (Bergom et al. 2005). Although the exact mechanism by which PECAM-1 mediates cytoprotection has not been fully defined, palmitoylation has been shown

to play a critical role. Specifically, palmitoylation on Cys-595 by DHHC21 is required for PECAM-1 constitutive association with membrane microdomains and PECAM-1-mediated cytoprotection, where it may act as a crucial regulator of signaling and apoptosis events (Sardjono et al. 2006).

3.3.9 LYPLA1 (APT1) and LYPLA2 (APT2)

Chronic lymphocytic leukemia (CLL) cells are characterized by their resistance to CD95 (Fas/APO-1)-mediated apoptosis. As a result, CLL patients experience a progressive accumulation of long-lived and well-differentiated clonal B lymphocytes in peripheral blood, lymphoid tissue, and bone marrow. Efficient CD95 membrane localization in specialized domains and formation of stable receptor aggregates require palmitoylation (Feig et al. 2007); further, membrane proteins are significantly less palmitoylated in CLL cells compared with normal B cells (Berg et al. 2015). Recently, Berg and colleagues identified *LYPLA1* and *LYPLA2* as targets of the microRNAs, miR138a and miR424, which are downregulated in CLL. Consequently, *LYPLA1* and *LYPLA2* are overexpressed and interact directly with CD95 to promote its depalmitoylation, thereby impairing CD95-mediated apoptosis (Berg et al. 2015).

As noted earlier, Scrib is a scaffolding protein that organizes key signaling complexes to specify basolateral cell polarity and suppress aberrant growth. Scrib mislocalization from cell-cell junctions to the cytosol correlates with enhanced growth signaling and malignancy in many human cancers and can be triggered by inhibition of palmitoylation by DHHC7 (Chen et al. 2016). Scrib displacement from the PM has also been reported in benign epithelial cells when the EMT transcription factor, Snail, is expressed. Snail targets the Scrib palmitoylation cycle by repressing several PATs and elevating the expression and activity of APT2. Inhibition of APT2 restores balance to the Scrib palmitoylation cycle, promoting membrane relocalization and growth attenuation (Hernandez et al. 2017). Importantly, changes in APT1 abundance have also been shown to promote cancer, including changes in the transcriptional signatures of TNBC cells and the invasive behavior of melanoma cells (discussed in Sect. 3.4.4).

From these examples, it is clear that disrupting the homeostatic balance of protein palmitoylation causes significant and deleterious effects on normal physiology, and further identification of the substrates of each of these PATs and APTs will provide important information concerning the molecular mechanisms underlying cancer as well as reveal novel targets for pharmacologic intervention. Further, the development of specific PAT/APT protein inhibitors would provide vital reagents with which to study the pathophysiological importance of many palmitoylated proteins, including those mentioned above that may offer potential therapeutic benefit in cancer.

3.4 Regulatory Effects of *S*-Palmitoylation on Cancer-Related Signaling Proteins

S-Palmitoylation can regulate target protein functionality in several different ways—through membrane localization of regulatory proteins, membrane trafficking, subcellular targeting within the cell, modulating protein conformation, and impacting protein-protein interactions or protein complex formation. Because *S*-palmitoylation can dynamically regulate protein functionality, disruption in the homeostatic balance of protein palmitoylation can have significant and deleterious effects on signaling networks, resulting in an increase in tumorigenesis and metastasis. Several key examples of cancer-related signaling proteins that are regulated by palmitoylation are described in the following paragraphs and provide context for the growing importance of this posttranslational modification in cancer.

3.4.1 Ras Signaling

Members of the Ras gene family encode monomeric guanosine triphosphatases (GTPases) that transduce extracellular signals to the intracellular effector pathways that regulate cell proliferation, differentiation, and survival (Ulku and Der 2003). Ras-activating mutations that cause dysregulation in these pathways have been detected in approximately 30% of all human cancers, including solid tumors and hematologic malignancies (Ahearn et al. 2011). H-Ras, N-Ras, and the splice variants K-Ras4A and K-Ras4B are four widely expressed isoforms of Ras that are preferentially associated with specific cancer types. Activating mutations of *NRAS* are most common among myeloid malignancies, including acute myeloid leukemia, chronic myelomonocytic leukemia, and myelodysplastic syndrome (Cuiffo and Ren 2010). *HRAS* mutations predominate in head and neck squamous cell carcinoma but are relatively rare. Mutations in *KRAS* occur most frequently and are associated with pancreatic ductal adenocarcinoma (Cox et al. 2014).

All Ras proteins undergo a series of posttranslational modifications for differential targeting to distinct membrane subdomains, including farnesylation as a required first step and palmitoylation to stabilize their membrane attachment. H-Ras, N-Ras, and K-Ras4A are modified by palmitoylation of cysteines proximal to the C-terminal cysteinyl site of farnesylation (Linder and Deschenes 2007). Specifically, H-Ras is palmitoylated at Cys-181 and Cys-184 and N-Ras at Cys-184, whereas K-Ras4B is not further modified with lipids but contains a hexalysine, polybasic domain that interacts electrostatically to anionic phospholipids in the PM (Hancock et al. 1990). K-Ras4A possesses a dual membrane-targeting motif that includes a palmitoylated cysteine and a polybasic region making it unique among the four Ras proteins (Cox et al. 2014). These lipid modifications provide unique information for spatial organization and trafficking of the Ras proteins and hence are required for their normal function and for their cancer-promoting actions (Pedro et al. 2017).

For both H-Ras and N-Ras, *S*-palmitoylation occurs in the Golgi apparatus, stably associating the protein with the membrane and facilitating its vesicular transport

to the PM. Depalmitoylation leads to release of the protein from the PM and its return to the Golgi where it is kinetically trapped again by palmitoylation. Interrupting this transport cycle by preventing either palmitoylation or depalmitoylation mislocalizes Ras from the PM; therefore, PATs that mediate the attachment of palmitate or APTs, which mediate its removal, have become potential therapeutic targets for Ras-driven tumorigenesis (for a recent review, see Lin et al. (2017).) In mammalian cells, *S*-palmitoylation of H-Ras is mediated by DHHC9/GCP16 (Young et al. 2014; Lobo et al. 2002; Swarthout et al. 2005), while the APT1 and APT2 enzymes catalyze its depalmitoylation. APT1 appears to be engaged in regulation of the acylation-deacylation cycle not only of H-Ras but also several other cytoplasmic proteins including Gα subunits, endothelial nitric oxide synthase (eNOS), and SNAP-23 (Yang et al. 2010; Tomatis et al. 2010; Zeidman et al. 2009; Dekker et al. 2010). APT2 is 68% homologous to APT1 (Toyoda et al. 1999) but catalyzes the depalmitoylation of semisynthetic N-Ras more efficiently than APT1 (Rusch et al. 2011). More recently, a novel protein depalmitoylase named ABHD17 was found to deacylate N-Ras and mediate its relocalization to internal membranes (Lin and Conibear 2015; Yokoi et al. 2016).

Attempts have been made to inhibit these enzymes as a way to inhibit Ras-associated malignancies. To date, no compounds that selectively inhibit DHHC PATs have been developed, but small molecules that specifically inactivate DHHC9-GCP16 hold promise. DHHC9 promotes Ras PM localization and transformation of mammalian cells when overexpressed, suggesting that DHHC9 palmitoylates Ras proteins in a highly selective manner. Indeed, *ZDHHC9* knockout mice demonstrated a reduced ability of oncogenic N-Ras to transform bone marrow cells lacking DHHC9 and slowed leukemia progression and increased survival in a transplantation model (Liu et al. 2016). However, *S*-palmitoylation of N-Ras was not completely reduced in the bone marrow cells from the $Zdhhc9^{KO}$ mice, suggesting that N-Ras may be palmitoylated by other PATs. Palmostatin B (Palm B) is the first compound shown to inhibit Ras depalmitoylation in cells (Dekker et al. 2010). Exposing transduced hematopoietic cells and bone marrow from N-Ras- and K-Ras-mutant mice to Palm B had similar effects on protein localization and colony growth; however, Palm B blocked the cytokine-independent growth of hematopoietic cells expressing oncogenic N-Ras, but not mutant K-Ras (Xu et al. 2012). Palm B also reduced proliferation and increased apoptosis in melanoma cell lines expressing oncogenic forms of N-Ras (Vujic et al. 2016). Notwithstanding, inhibiting the oncogenic effects of N-Ras in leukemia will require identifying and inhibiting additional depalmitoylation enzymes as recent studies have shown that APT1 and APT2 are not the only Palm B targets as was once believed (Lin and Conibear 2015; Vujic et al. 2016).

Targeting Ras palmitoylation may also be effective in treating K-Ras4A-associated malignancies. Several reports have shown that altered K-Ras4A/K-Ras4B ratios correlate with progression of lung and colorectal adenocarcinoma (Patek et al. 2008; Plowman et al. 2006) and that K-Ras4A plays an important role in lung carcinogenesis (To et al. 2008). Mutation at the palmitoylation site of oncogenic K-Ras4A significantly abrogated its leukemogenic potential, and palmitoylation-defective

K-Ras4A induced leukemia in mice but with a longer latency (Zhao et al. 2015). N-Ras/K-Ras4A chimeric constructs demonstrated that the KIKK motif of K-Ras4A contributes to K-Ras4A oncogenic activity and that mutations at both the palmitoylation site and the KIKK motif completely abolish the ability of oncogenic K-Ras4A to induce leukemogenesis in mice (Zhao et al. 2015). Thus, targeting Ras palmitoylation and the KIKK membrane-targeting motif may be a potential treatment strategy for K-Ras4A-associated malignancies.

3.4.2 EGFR Signaling

EGFR belongs to the ErbB family of receptor tyrosine kinases (RTKs), which respond to extracellular growth cues by initiating downstream signaling cascades through various effector pathways. Mutations in EGFR that lead to its constitutive activation and subsequent uncontrolled cell growth are detectable in a variety of human cancers, including NSCLC (Normanno et al. 2006) and breast cancer (Roskoski Jr. 2014); moreover, EGFR overexpression correlates with cancer progression, metastasis, and poor prognosis (Hynes and Lane 2005). Therapeutic approaches aimed at targeting EGFR include tyrosine kinase inhibitor (TKI) therapy and monoclonal antibody inhibitors; however, these anti-EGFR reagents have had limited clinical success with many patients developing resistance within a few months of treatment (Weiss 2012).

Recent studies suggest that targeting *S*-palmitoylation of the EGFR receptor may be a viable strategy to improve EGFR-based cancer therapy. Two separate studies demonstrated that palmitoylation of EGFR is required for its signaling activity, albeit at different sites (Bollu et al. 2015; Runkle et al. 2016). Cys-1025, Cys-1034, and Cys-1122 within the C-terminal tail of EGFR were shown to be palmitoylated by DHHC20, and *ZDHHC20* silencing increased EGFR activation and the dependency on EGFR signaling for cancer cell survival (Runkle et al. 2016). In a subsequent study, *ZDHHC20* silencing increased cell death induced by the EGFR inhibitor gefitinib in K-Ras- and EGFR-mutant cell lines via Cys-1025, but not in MCF7 cells harboring wild-type K-Ras. *ZDHHC20* silencing also increased sensitivity to gefitinib-induced cell death in lung cancer cells harboring the EGFR inhibitor-resistant mutation, T790M (Kharbanda et al. 2017). These data suggest that targeting DHHC20 in combination with TKI therapy may be an effective approach to overcoming cancers inherently resistant to EGFR targeted therapy.

3.4.3 MET Signaling

c-Met is a receptor tyrosine kinase activated by its ligand, hepatocyte growth factor (HGF), which triggers a spectrum of MET-driven growth activities that promote embryonic development, tissue remodeling, and cancer progression. Aberrant activation of c-Met signaling can cause uncontrolled proliferation and survival, as well as enhanced invasive potential (Coleman et al. 2016); thus, inhibiting c-Met activity

has become a targeted approach for preventing cancer progression and for overcoming therapeutic resistance (Steffan et al. 2011). Recently, *S*-palmitoylation has been shown to regulate the intracellular trafficking and stability of c-Met. Inhibition of palmitoylation reduced the expression of c-Met in multiple cancer cell lines post-transcriptionally and led to its accumulation at the Golgi. Two cysteines were identified as putative palmitoylation sites, Cys-624 and Cys-894, but neither site when mutated completely abolished palmitoylation, surface expression, or c-Met levels, suggesting that multiple cysteine residues may be required for proper trafficking of c-Met. Because palmitoylation is required for c-Met transport from the Golgi to the PM, targeting this modification may serve as a therapeutic modality for c-Met-driven tumorigenesis (Steffan et al. 2011).

3.4.4 Wnt Signaling

While signaling by Wnt proteins regulates a wide range of developmental and physiological process such as cell differentiation, polarity, and migration, dysregulation of the Wnt pathway promotes the initiation and progression of many human cancers, especially CRCs (Clevers and Nusse 2012). Canonical Wnt signaling via β-catenin is transduced by two receptor families, the Frizzled proteins and lipoprotein receptor-related proteins (LRPs) 5 and 6. Palmitoylation of LRP6 is required for its exit from the endoplasmic membrane to the cell surface; moreover, palmitoylation renders LRP6 dependent on Lys-1403, likely through its ubiquitination, to mediate Wnt signaling (Abrami et al. 2008). While the underlying mechanism is not clear, palmitoylation of LRP6 may enable its association with Casein kinase γ, which is required for LRP6 signal transduction.

Production of active Wnt ligand depends on attachment of palmitoleate to a conserved serine by the MBOAT acyltransferase Porcupine (Porcn). Recent studies demonstrate that Porcn overexpression promotes palmitoylation of Wnt1 and enhances the ability of Wnt to promote Wnt1 trafficking to the cell surface as well as secretion (Galli et al. 2016). Given that palmitoylation of Wnt is required for its signaling activity, Porcn inhibitors have been explored as potential chemotherapeutics in Wnt-driven cancers (for a recent review, see Resh (2017)). LGK974 (e.g., WNT974 (Zhang and Lum 2016)) is a Porcn inhibitor in Phase I clinical trials that has been shown to directly inhibit the enzymatic activity of Porcn using a novel in vitro fatty acylation assay (Asciolla et al. 2017). LGK974 inhibited Wnt peptide acylation in a dose-dependent manner with an IC_{50} = 12.9 nM which was similar to its IC_{50} = 7.5 nM in a luciferase-based reporter assay of Wnt signaling. Importantly, this assay also revealed that Porcn and hedgehog acyltransferase (Hhat) are dedicated acyltransferases for Wnt and Shh proteins, respectively (Asciolla et al. 2017).

Like canonical Wnt signaling, the noncanonical Wnt pathway has also been linked to cancer progression and metastasis. Wnt5a, the noncanonical Wnt ligand, is increased in several human cancers such as melanoma, colon, and gastric cancer and correlates with poorer outcome (Bakker et al. 2013; Kurayoshi et al. 2006; Saitoh et al. 2002; Weeraratna et al. 2002). Wnt5a signaling induces asymmetric

localization of the melanoma cell adhesion molecules (MCAM). Wang et al. showed that Wnt5a promotes rapid depalmitoylation of the cell adhesion molecules MCAM and CD44, causing MCAM asymmetry and increased collagen invasion (Wang et al. 2015). This phenotype was blocked by inhibiting the depalmitoylating enzyme APT1 with shRNA or Palm B and restored by APT1 overexpression; however, overexpression of APT1 could affect other palmitoylated proteins that affect cell adhesion or cell motility. Notably, a point mutation blocking MCAM palmitoylation on Cys-590 mimicked the effects on Wnt5a signaling on MCAM localization and invasive melanoma cell behavior. Wnt5a may modulate APT1 function through the disruption of an inhibitory interaction with Dvl2, causing spatial changes in MCAM palmitoylation. This study also implicated DHHC20 as playing a potential role in the palmitoylation of MCAM, as cells expressing mutant forms of DHHC20 had reduced levels of palmitoylated MCAM and mutations in *ZDHHC20* (and *ZDHHC15*) have been reported in metastatic melanoma (Berger et al. 2012; Stark et al. 2011) and ovarian carcinoma.

APT1 has also recently been shown to direct the asymmetric localization of Numb and β-catenin in human cancer cell lines derived from malignant tumors, such as TNBC and osteosarcomas (Stypulkowski et al. 2018). Reciprocal interactions between APT1 and the Rho family GTPase CDC42 promoted the asymmetric localization of Numb and β-catenin to the PM, restricting Notch- and Wnt-responsive transcriptional activity to one daughter cell. Further, APT1 abundance changed the transcriptional signatures of MDA-MB-231 TNBC cells, similar to changes in Notch- and β-catenin-mediated Wnt signaling (Stypulkowski et al. 2018). This suggests that APT-mediated depalmitoylation is an important mechanism that regulates asymmetric partitioning of Notch and Wnt signaling during cell division.

3.4.5 Sonic Hedgehog (Shh) Signaling

Hedgehog (Hh) signaling regulates several developmental processes including organogenesis and tissue patterning and differentiation of human thymocytes and bone remodeling in adults. However, various types of human cancers display aberrant Hg signaling, including medulloblastoma, gastrointestinal tumors, and prostate (Beachy et al. 2004) and pancreatic cancer (Thayer et al. 2003). The Hh family of secreted morphogens comprising Sonic (Shh), Indian (Ihh), and Desert Hedgehog (Dhh) mediate Hh signaling in humans. Proper functioning of these proteins requires dual posttranslational lipidation via a cholesteryl ester at the C-terminal carboxylate and a palmitoyl amide at the N-terminal amine (Pepinsky et al. 1998). For mature Shh, the first six amino acids are recognized by Hhat—an MBOAT protein that *N*-palmitoylates Hh proteins, which is essential for ligand activity (Hardy and Resh 2012); thus, inhibiting Hhat may be an effective means for blocking Hh signaling (Matevossian and Resh 2015; Petrova et al. 2015).

The role of Hhat as a therapeutic target in Hh-driven tumorigenesis has been reviewed recently (Resh 2017). Small-molecule inhibitors targeting Hhat have been identified, including RUSKI-43, which blocked Hhat-mediated Shh palmitoylation

in vitro and in cells (Petrova et al. 2013). Rodgers et al. recently characterized this series of dihydrothienopyridines for anti-Hhat activity using orthogonal cell-based assays and found that RUSKI-43, the lead compound in the series, possessed off-target cytotoxic activity, masking its effect on Hhat-dependent signaling. However, RUSKI-201 displayed specific inhibition of Hhat in cells with no off-target cytotoxicity, suggesting that it is an ideal chemical probe for inhibition of Hhat catalytic function (Rodgers et al. 2016). Thus, RUSKI-201 may improve the effectiveness of other Hh signaling inhibitors when used in combination (Lee et al. 2014; Rhim et al. 2014).

3.4.6 Apoptotic Signaling

Defects in apoptotic pathways can promote cancer development and cause cancers to become resistant to chemotherapy. Several key proteins that regulate apoptosis have been identified as being palmitoylated. As mentioned previously, the cell adhesion and signaling molecule PECAM-1, which is palmitoylated by DHHC21, has been shown to potently suppress apoptosis in a variety of cellular systems. Notably, palmitoylation by DHHC21 is required for PECAM-1 constitutive association with membrane microdomains and PECAM-1-mediated cytoprotection, where it may act as a crucial regulator of signaling and apoptosis events (Sardjono et al. 2006).

BCL-2-associated X protein (BAX) is the main regulator of the intrinsic pathway of apoptotic cell death, driving the mitochondrial outer membrane permeabilization (MOMP) and the concomitant release of cytochrome *c* into the cytosol. Dysregulation of this process can lead to numerous diseases, cancer being among them. BAX's ability to associate with mitochondria is a critical regulatory step in its ability to insert into the mitochondrial outer membrane (MOM) and trigger cytochrome *c* release, which is regulated by *S*-palmitoylation at Cys-126 (Frohlich et al. 2014). In primary tissues and cultured cells, reduced BAX palmitoylation inhibited its mitochondrial translocation, its oligomerization, caspase activity, and apoptosis, while overexpression of specific PATs increased BAX palmitoylation and accelerated apoptosis. Once captured by the MOM, the palmitoyl moiety may orient Bax at the mitochondrial surface facilitating integration of helices α5 and α6 into the membrane. *S*-Palmitoylation may also facilitate BAX dimer formation at the mitochondrial surface and assembly of higher-order oligomers that facilitate pore formation and release of proapoptotic proteins into the cytosol. Importantly, malignant tumor cells with reduced BAX-mediated proapoptotic activity also showed reduced *S*-palmitoylation of BAX at Cys-126 (Frohlich et al. 2014).

Apoptotic calcium release by PM- and ER-resident calcium channels is a critical regulator of cell death, and growing evidence suggests that defects in this pathway contribute to cancer development; moreover, *S*-palmitoylation has been shown to regulate apoptotic calcium signaling. Components of the T cell receptor (TCR) complex, such as Lck, Zap-70, PLC-γ1, and other TCR components, have been shown to be required for apoptotic calcium release in T cells after engagement of the FasR with FasL (Akimzhanov et al. 2010). Within minutes of Fas receptor

engagement, there is a rapid increase in Lck palmitoylation by PM-localized DHHC21 and partitioning of Lck into lipid rafts where it interacts with the TCR complex and triggers downstream apoptotic calcium release (Akimzhanov and Boehning 2015). Although a specific Lck depalmitoylating enzyme has yet to be defined, it is possible that these enzymes could be directly or indirectly regulated by calcium ions. Importantly, several of the proliferative and pro-metastatic signaling pathways already mentioned like Wnt and Ras signaling are calcium dependent and regulated by *S*-palmitoylation (Chen and Boehning 2017).

VPAC1 is a receptor belonging to class B GPCRs and shared by two ligands: pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP). Various malignant epithelia-derived tumors and their metastases are associated with elevated VPAC1 expression including breast (Valdehita et al. 2012), prostate (Fernandez-Martinez et al. 2012), lung, and colorectal carcinomas (Liu et al. 2014). In a recent study by Yu and colleagues, the Cys-37 in the N-terminal extracellular domain of VPAC1 was shown to be palmitoylated. CHO cells expressing a palmitoylation-incompetent form of VPAC1 showed higher proliferative activity than those expressing wild-type VPAC1, which displayed higher anti-apoptotic activity (Yu et al. 2017). While treatment with 2-BP significantly inhibited nuclear translocation of wild-type VPAC1 and its anti-apoptotic activity, it remains questionable whether palmitoylation of Cys-37 is directly involved, as 2-BP is not an exclusive inhibitor of palmitoylation (Davda et al. 2013).

3.4.7 Metastasis

More than 90% of the morbidity and mortality associated with cancer is due to metastasis, which is the ultimate step in a tumor cell's progression toward autonomy from the host (Stafford et al. 2008). Acquiring the ability to break free from the primary tumor is one of the earliest events in the metastatic cascade and requires coordinated activation of many cell adhesion signaling cascades. CD44 is an adhesion protein that regulates cell migration and is sequestered in cholesterol-rich lipid rafts by palmitoylation of two cysteines adjacent to its TM domain, which are required for its ability to endocytose its ligand hyaluronan (Thankamony and Knudson 2006). In a study by Babina et al., mutation of these palmitoylation sites reduced CD44 raft association in invasive MDA-MB-231 cells and enhanced their migration; moreover, expression of palmitoylation-incompetent (raft-excluded) CD44 mutants in noninvasive MCF-10a cells reversibly induced the phenotypic appearance of EMT and increased cell motility (Babina et al. 2014). This study also showed that levels of palmitoylated CD44 were lower in invasive ductal carcinomas with less lipid raft marker colocalization compared to non-tumor tissue. Thus, these results support a mechanism whereby CD44 palmitoylation and lipid raft association inversely regulate breast cancer cell migration (Babina et al. 2014).

Claudins comprise a family of closely related four-pass TM proteins that mediate cell adhesion via tight junctions in a calcium-independent manner. In addition to cell adhesion, claudins are also involved in the regulation of cell permeability and

are crucial components of signaling pathways that regulate epithelial proliferation and differentiation (Rinker-Schaeffer et al. 2006). Claudins have their N- and C-termini on the interior and have membrane-proximal cysteines in similar locations near the ends of the second and fourth TM domains that are palmitoylated (Lynch et al. 2007; Van Itallie et al. 2005). Claudin-7 (cld7), a tight junction (TJ) component, is also found in the cytoplasm and basolaterally where it associates with EpCAM (EpC) in the glycolipid-enriched membrane domains (GEMs). EpC-cld7 colocalization is associated with metastasis in colorectal (Kuhn et al. 2007) and pancreatic cancer (Thuma and Zoller 2013). Importantly, *S*-palmitoylation of cld7 prohibits tight junction integration and promotes recruitment into GEMs, where it associates with integrins and cytoskeletal linker proteins that promote motility and invasion (Heiler et al. 2015). To search for the mechanism whereby GEM-localized and palmitoylated cld7 promotes metastasis, Thuma and colleagues examined the metastatic capacity of the rat pancreatic adenocarcinoma cell line ASML following cld7 knockdown or rescue with a mutated palmitoylation site (cld7mPalm). Both cld7 knockdown and cld7mPalm ASML cells had reduced motility and invasiveness, due to cld7 association with alpha6beta4, ezrin, uPAR, and MMP14, which jointly support motility and invasion. Further, GEM-localized, palmitoylated cld7 was shown to associate with components of vesicle transport machineries involved in exosome biogenesis (Thuma et al. 2016).

3.5 Targeting *S*-Palmitoylation in Cancer

Our ability to understand *S*-palmitoylation and its importance to human health and disease is only as good as the technological methods we use to make accurate and valid measurements. Recent methodological advances have improved our ability to annotate palmitoylated proteins and to identify PAT (and APT)/substrate pairs, yet a framework for predicting PAT/APT substrate selectivity is lacking, and our understanding of how palmitoylation affects cancer-related signaling processes is still unclear. Progress in the field has also been hindered by a lack of pharmacological inhibitors of *S*-palmitoylation, previously relying on generic lipid-based compounds such as 2-BP, tunicamycin, and cerulenin with multiple, undesired off-target effects. However, selective and potent inhibitors of PATs, like those recently developed for APTs, would provide vital reagents with which to study the pathophysiological importance of many palmitoylated proteins implicated in tumorigenesis and may offer potential for therapeutic development.

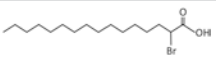
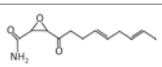
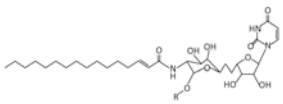
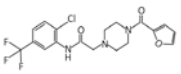
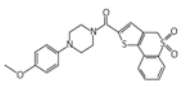
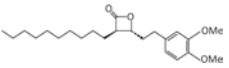
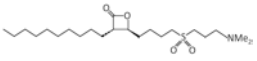
3.5.1 PAT Inhibitors

In recent years, the development of pharmacological modulators of PATs has garnered increasing interest due to the prospect of altering the localization and activity of palmitoylated proteins, several of which are involved in pathological processes like cancer. Lipid-based PAT inhibitors have been featured extensively in the

literature and include cerulenin, tunicamycin, and the palmitate analogue, 2-BP (Table 3.1). Sometimes referred to as a "specific" inhibitor of palmitoylation, 2-BP has been widely used as an inhibitor of PAT-mediated palmitoylation that covalently (and irreversibly) modifies the enzyme active site in vitro (Fukata et al. 2004; Jones et al. 2012; Leong et al. 2009; Resh 2006; Webb et al. 2000; Jennings et al. 2009); however, it has been shown to non-selectively inactivate other membrane-bound enzymes (Coleman et al. 1992), including several enzymes involved in lipid metabolism (Chenette et al. 2005) and the deacylating enzymes, APT1 and APT2 (Pedro et al. 2013). Recent studies discredit the use of 2-BP as an exclusive inhibitor of enzymatic *S*-palmitoylation because of its promiscuity (Davda et al. 2013).

Screening for newer selective inhibitors to accurately establish the role of individual PATs in regulating protein palmitoylation and the resulting downstream effects is essential in light of the non-specificity of 2-BP and other lipid-based inhibitors. A lack of suitable assays for high-throughput screening, as well as insufficient knowledge of fundamental aspects of PAT enzymes including their mechanism of catalysis and acyl-CoA binding and recognition, has hindered such efforts.

Table 3.1 Inhibitors of palmitoyl acyltransferases (PATs) and acyl-protein thioesterases (APTs)

Inhibitor	Structure	Proposed mechanism of action
2-bromopalmitate		Lipid-based; non-specific; irreversibly inhibits PAT autoacylation
Cerulenin		Lipid-based; non-specific; inhibits palmitoylation by alkylating cysteine residues in PATs or their substrates
Tunicamycin		Lipid-based; non-specific; inhibits N-linked glycosylation and may compete with palmitoyl-CoA for binding to PATs
Compound V [2-(2-hydroxy-5-nitro-benzylidene)-benzo[*b*] thiophen-3-one]		Reversibly inhibits PAT autoacylation; non-specific
ML348		Selective APT1 inhibitor (K_i = 280 nM)
ML349		Selective APT2 inhibitor (K_i = 120 nM)
Palmostatin B		Potent inhibitor of APT1/APT2 that covalently modifies and inactivates the active site serine (IC_{50} = 120 nM); not selective between isoforms
Palmostatin M		Mechanism based inhibitor of APT1/APT2 that covalently modifies and inactivates the active site serine; not selective between APT isoforms

However, a recent study by Rana et al. has revealed the crystal structures of two DHHC family members—human DHHC20, a catalytically inactive DHHC15 mutant from zebrafish (zfDHHS15), and human DHHC20 conjugated to an irreversible inhibitor mimicking the acylated enzyme intermediate—representing a major breakthrough in the field (Rana et al. 2018). Their results show that the four TM helices of hDHHC20 and zfDHHS15 adopt a tepee-like structure in the membrane with the active site at the membrane-cytosol interface where substrate engagement and catalysis occur. The CRD binds two zinc ions, which likely provide structural stability, but do not actively coordinate the nucleophilic cysteine. The TM domain forms a cavity that accommodates the acyl chain of acyl-CoA, with residues within the cavity responsible for fatty acyl recognition and chain-length selectivity. The C-terminal domain forms a supporting structure for the TM domain and consists of an amphipathic helix and a hydrophobic loop and contains conserved motifs that interact with the active site. Importantly, these detailed structures will foster the discovery and development of structure-based PAT inhibitors.

Novel methodologies for palmitoylation assays that are amenable to higher-throughput compound screening have been emerging (see review by Draper and Smith, 2009 (Draper and Smith 2009)). Smith and colleagues used a cell-based assay approach to screen a compound library to identify more selective inhibitors of palmitoylation (Ducker et al. 2006). The screen utilized, among other assays, cell-permeable GFP-linked substrate peptides to observe the effects of each compound in inhibiting the localization of such substrates to the PM. Five lead compounds, designated compounds I–V, were identified that inhibited cellular processes associated with palmitoylation. However, follow-up studies revealed that just one of the compounds, compound V (or 2-(2-hydroxy-5-nitro-benzylidene)-benzo[*b*]thiophen-3-one), inhibited the activity of all four DHHC proteins that were tested (Jennings et al. 2009). It was also determined that compound V inhibits PAT autoacylation, a common property of all known PATs (Mitchell et al. 2006), in the same way that 2-BP does; thus, it would not be selective for different PATs. However, unlike 2-BP, inhibition by compound V was largely reversible. Consequently, given that 2-BP is also known to inhibit other enzyme families, compound V is a good candidate for future structure-activity tuning studies to increase selectivity and potency.

More recently, several high-throughput approaches to identify specific inhibitors of *S*-palmitoylation have emerged that focus on therapeutically relevant targets, including the oncogene Ras. As mentioned earlier, there are four known Ras proteins—H-Ras, N-Ras, and the splice-variants K-Ras4A and K-Ras4B—all of which require palmitoylation for stable membrane anchoring. Ganesan and colleagues developed a click chemistry-based, high-throughput platform to screen for small molecules that inhibit Ras palmitoylation (Ganesan et al. 2017). Their approach utilized a truncated synthetic peptide comprised of the minimal membrane-anchoring region of N-Ras containing the cysteine-181 palmitoylation site as well as the primary C-terminal farnesyl group to ensure specificity. Counter-screening was performed using an unrelated target peptide that contained the minimal membrane-anchoring sequence, the cysteine-3 and cysteine-6 palmitoylation sites, and the primary N-terminal myristoyl group of the SFK, Fyn. Both peptides were

enzymatically palmitoylated in vitro, and known palmitoylation inhibitors were readily detected using a 384-well plate format (Ganesan et al. 2017). Hence, this integrated platform to screen and identify specific regulators of palmitoylated proteins may have applicability to therapeutically relevant targets involved in cancer.

One major consideration regarding the design of PAT inhibitors is the specificity of PAT/substrate recognition. Even though the substrate pool overlaps for all the known PATs, evidence from knockout animal models demonstrates that loss-of-function of a single PAT can have dramatic pathological effects. Presumably, this is the direct result of hypo-palmitoylation of a combination of multiple substrates consisting of PAT-specific substrates and more common (i.e., substrates palmitoylated by multiple PATs) target substrates. However, given the homology of the DHHC protein active sites (Mitchell et al. 2006), specificity of palmitoylation must be derived in part from other unique physical interactions of individual PATs with their substrates. It is known that the sequence of amino acids surrounding a substrate cysteine partially defines the potential for that cysteine to be palmitoylated. However, the physical determinants for substrate recognition likely extend throughout the accessible portions of the PAT and substrate, as is the case for DHHC17 (Huang et al. 2009). The AR domain of both DHHC17 and DHHC13 recognizes evolutionary-conserved and closely related zDABM sequences (Lemonidis et al. 2015). Peptide array-based screening using the zDABM binding motif of synaptosomal nerve-associated protein 25 (SNAP25) and cysteine string protein α (CSPα) validated 95 human zDABM sequences distributed among 90 proteins, of which 62 were novel putative DHHC17-interacting proteins (Lemonidis et al. 2017). Similar approaches to identify and characterize substrates for sequence patterns and other potential predictors of activation via specific PATs will be an important future step for development of selective PAT inhibitors.

Another complication of PAT-substrate recognition lies in that each PAT can traverse the membrane multiple times. Thus, it is logical to assume that beyond sequence alone, the local membrane environment (i.e., exposed/accessible regions of each PAT) is important for determining in vivo PAT structure and substrate recognition. However, several PATs have been purified from membrane that can remain enzymatically active (Jennings et al. 2009), so it may be possible to use enzyme activity-based and drug-binding screens for selective PAT inhibitors. Additionally, other factors that are likely to regulate palmitoylation are the temporal and spatial aspects of PAT and substrate expression.

3.5.2 APT Inhibitors

As mentioned previously, APT function has not been characterized as extensively as PATs. APT1 and APT2 have been reported to have depalmitoylating activity against diverse palmitoylated substrate proteins in vitro (Tomatis et al. 2010; Duncan and Gilman 1998; Hirano et al. 2009; Tian et al. 2012), making them attractive targets for pharmacologic intervention; moreover, recent studies have identified the membrane-anchored ABHD17 proteins as depalmitoylating enzymes that

specifically regulate N-Ras and PSD-95 and MAP6 palmitoylation cycles in mammalian cells (Lin and Conibear 2015; Yokoi et al. 2016; Tortosa et al. 2017).

The availability of the APT1 and APT2 crystal structures has enhanced the discovery of APT inhibitors (Won et al. 2016; Devedjiev et al. 2000). One of the first efforts to identify novel APT inhibitors was based on the commercially marketed weight loss drug tetrahydrolipstatin (THL or Orlistat), a prototypical serine hydrolase inhibitor, following its observed structural homology with gastric lipase (Dekker et al. 2010). THL possesses an electrophilic β-lactone moiety that covalently inactivates certain serine hydrolases (Hadvary et al. 1991). Derivatization of the β-lactone scaffold and screening for inhibition against APT1 led to the discovery of Palmostatin B or (APT1 IC_{50} = 5.4 nM) (Fig. 3.2), the first compound shown to inhibit Ras depalmitoylation in cells and reversion of malignant phenotypes (Dekker et al. 2010). Further optimization yielded a more potent and more soluble analogue, Palmostatin M (APT1 IC_{50} = 2.5 nM) (Table 3.1) (Hedberg et al. 2011). Both Palm B and Palmostatin M are mechanism-based inhibitors of APTs that covalently modify and inactivate the active site serine residue. Although both compounds display high selectivity for APTs among other cellular lipid esterases, neither is selective between the two APT isoforms, APT1 and APT2 (Dekker et al. 2010; Hedberg et al. 2011).

With the aim of developing APT isoform-specific inhibitors, a substrate-free methodology called fluopol-ABPP (Bachovchin et al. 2009) was used to screen a large compound library (315,004 compounds) against APT1 and APT2 in parallel, and the resulting lead compounds were then selected for downstream gel-based ABPP assays (Adibekian et al. 2012). Eventually two compounds, containing a piperazine amide motif linked to a five-membered heterocycle and designated ML348 and ML349, were identified as potent and selective inhibitors of APT1 and APT2, respectively (Table 3.1); moreover, the compounds were reported to maintain in vivo potency and selectivity in mice, thus representing valuable tools for future studies toward profiling dynamic protein palmitoylation as well as individually establishing the roles of APTs (Adibekian et al. 2012, 2010a, b). Other inhibitors of APT1/APT2 have been reported, including triazole urea (ML211) and the N-hydroxyhydantoin carbamate (ML378); however, neither is selective (Won et al. 2018). Notably, the co-crystal structures of APT1 and APT2 binding to their respective selective inhibitors have been solved, and each adopts a distinct conformation within each active site. Further analyses suggest that the inhibitors block access to the catalytic triad and occlude the putative acyl-binding pocket, providing insight into the mechanism of APT-substrate specificity and setting the stage for inhibitor optimization (Won et al. 2016).

3.6 Conclusion

The importance of PAT-/APT-mediated *S*-palmitoylation/depalmitoylation as a dynamic regulator of protein function has only recently been discovered, particularly in the milieu of cancer-related signaling. Recent studies provide examples of the versatility of palmitoylation in modifying protein function beyond traditional

membrane association and targeting. Although several examples of cancer signaling proteins regulated by palmitoylation have recently emerged, we are likely only in the initial stages of uncovering the full extent and significance of such regulation. Notwithstanding, it is clear from the studies described in this chapter that disrupting protein palmitoylation homeostasis, in either direction, can have significant and detrimental effects on signaling networks that regulate tumorigenesis and metastasis. Having a better understanding of PATs/APTs and the corresponding substrates that contribute to cancer pathogenesis is key to identifying new targets for pharmacologic intervention. Indeed, the development of protein inhibitors that selectively target PATs or APTs would provide vital reagents with which to elucidate the functional importance of these PAT-substrate/APT-substrate relationships within the context of cancer signaling mechanisms and offer promise of potential new therapeutic strategies for cancer. Targeting those PATs for which overexpression is oncogenic may be the most logical ones to target first. More conclusive data from in vivo experiments that link PATs to tumorigenesis are warranted to motivate drug discovery programs on a large scale toward this goal.

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Advances in Mass Spectrometry-Based Proteomics and Its Application in Cancer Research

4

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Abstract

With the advent of high-resolution/high mass accuracy instrumentation, sophisticated informatic approaches, and advances in liquid chromatography, mass spectrometry-based proteomics has emerged as an indispensable and widely used tool for the identification, characterization, and quantification of proteins on a large scale. Deep proteome analyses can now sequence over 14,000 protein isoforms for a single human cell line rivaling the depth of next-generation RNA sequencing technology. Without additional enrichment steps, highly sensitive MS-based proteomic studies yield comprehensive identification of major post-translational modifications (PTMs). Isotopic labeling techniques enable the comparison of multiple samples in a single mass spectrometry experiment, while data-independent acquisition strategies provide comprehensive protein coverage and quantification against complex backgrounds.

Keywords

Mass spectrometry · Quantitative proteomics · Post-translational modifications · Cancer pathways · Isotopic labeling

Proteins are the essential mediators of cellular function: Their biological activities and interactions catalyze biochemical reactions and thereby facilitate physiological and pathological processes. Characterization of the functional state of proteins as well as measuring changes in protein abundances reveals fundamental insights into

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these processes and ultimately deepens our understanding of the underlying biochemistry and molecular biology. While gene expression analysis by real-time PCR or via transcriptome sequencing provides valuable insights into biological pathways, it can only infer protein abundance information. There is a growing consensus that correlation between mRNA and protein levels is in general modest (Gygi et al. 1999b; Schwanhäusser et al. 2011; Skelly et al. 2013; Lundberg et al. 2010). The protein phenotype appears to be buffered against transcriptional variation (Fu et al. 2009) Correlations of transcripts and proteins depend on cellular location and biological function (Conrads et al. 2005) and are controlled by tissue-specific post-transcriptional regulation (Franks et al. 2017). Therefore, direct measurements of proteins are preferable since they will more accurately reflect cellular status and provide insights into the molecular mechanisms that underlie physiological and pathological processes. Mass spectrometry-based proteomics has emerged as the method of choice for the identification, characterization, and quantification of proteins (Picotti et al. 2013; Aebersold and Mann 2016). Protein identification and characterization is critical to identify alternatively spliced proteins, proteolytic processing, and post-translational modifications that alter the composition and functional status of proteins at the post-transcriptional level. It is estimated that the diversity of the roughly 20,300 protein-coding genes is increased to over 500,000 proteoforms by alternative splicing and post-translational modifications (phosphorylation, glycosylation, proteolytic truncations) (Smith et al. 2013).

The ability to identify proteins at a large scale has been primarily driven by the advances in mass spectrometric instrumentation, informatic workflows, and separation of complex protein mixtures. Liquid chromatography (LC) and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) are two of the most commonly applied separation techniques prior to mass spectrometric analysis. Quantitative proteomics has developed into an indispensable tool for cancer research to analyze disease-related tissues and body fluids in order to identify proteins, protein post-translational modifications, or protein complexes that can be used to detect the disease early, prognose disease outcome, and monitor response to therapeutic intervention and for the elucidation of molecular mechanisms for the development of novel therapeutics. Oncoproteomics has been extensively reviewed, from proteomic studies of tumor tissue and cancer cell lines to profiling of plasma and other body fluids for cancer biomarkers (Huang et al. 2017; Belczacka et al. 2018; Tan et al. 2012; Cantor et al. 2015; Veenstra 2013; Faria et al. 2017). Here, we highlight the most promising quantitative proteomics approaches in the context of studying cancer signaling pathways.

4.1 Differential Analysis by 2D-PAGE

In 2D-PAGE, proteins are initially resolved by isoelectric focusing followed by a separation based on molecular mass. After protein staining, specialized image analysis software is used to identify differentially expressed protein spots. Spots

of interest are excised, and proteins are in-gel digested with exogenous proteases (i.e., trypsin). The resulting peptides are recovered and their molecular masses measured by mass spectrometry. In the early stages of proteomics, subsequent peptide identification was performed by peptide mass fingerprinting (PMF) using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), in which peptide masses were matched against theoretically predicted peptide masses in a database of candidate proteins (Monteoliva and Albar 2004). Nowadays, with the ubiquity of LC-MS/MS instrumentation, protein identification is typically performed in higher throughput and with higher accuracy through the matching of peptide fragmentation data obtained from tandem mass spectrometry experiments (MS/MS) to theoretically predicted peptide fragment masses. 2D-PAGE is a classical proteomics workflow that provides a straightforward visual and quantitative comparison of differences in protein composition. It has been extensively employed in cancer research, including for the detection of tumor-associated proteins in colorectal cancer tissue samples (Wang et al. 2007; Stulík et al. 1999; Xing et al. 2006) and in the combination with laser capture microdissection (LCM) (Shi et al. 2011). However, the application of 2D-PAGE has been declining in recent years due to its limitations in throughput and reproducibility, sensitivity, dynamic range, and its laborious nature (Belczacka et al. 2018). The detection limits of the most commonly used stains range from 500 ng/mm^2 (colloidal Coomassie Brilliant Blue) to 0.1 ng/mm^2 (silver stain and fluorescent dyes). Some of these drawbacks can be overcome by the usage of narrow-range IPG strips to increase the resolving power in the initial isoelectric focusing dimension. To increase reproducibility and improve comparative analyses, difference gel electrophoresis (DIGE) was developed, a form of multiplexed 2D-PAGE where up to three different protein samples are fluorescently labeled prior to gel separation. DIGE-based differential proteomics analysis has been successfully used in the discovery phase of cancer biomarker studies (colorectal, prostate cancer) when the proximal tissue samples are being analyzed at greater depth before validation of potential markers by ELISA in serum (Hamelin et al. 2011; Pang et al. 2010).

A particular strength of 2D-PAGE is the ability to detect and visualize proteoforms – the different molecular structures that the protein products of a single gen can assume due to genetic variations, alternatively spliced RNA transcripts, and post-translational modifications (PTMs) (Smith et al. 2013). PTMs including proteolytic processing, deamidation, glycosylation, acetylation, alkylation, cysteine oxidation, tyrosine nitration, and phosphorylation regulate many cellular signaling pathways. PTMs alter the molecular mass and/or the isoelectric point of the protein. For example, different phosphorylation states of a protein are observable as horizontal spot trains in a 2D gel, whereas glycosylation can alter both the pI and the molecular weight of proteins resulting in clusters shifted both horizontally and vertically (Löster and Kannicht 2008). ProMoST (http://proteomics.mcw.edu/promost.html) is a webtool that can be used to calculate gel shifts introduced by PTMs to facilitate more detailed analyses (Halligan et al. 2004).

4.2 Top-Down Proteomics

Ideally, intact proteins would be analyzed directly by mass spectrometry without the need for proteolytic digestion; protein identification, in turn, would be achieved by MS/MS fragmentation of the whole protein. As a liquid phase-alternative to 2D-PAGE, "top-down" proteomics has made substantial progress in the last decade, and it is now feasible to measure over 3000 proteoforms using a four-dimensional LC separation system that is integrated with high-resolution electrospray mass spectrometry analysis (Tran et al. 2011). However, similar to 2D-PAGE, the high complexity and dynamic range of protein concentrations encountered in proteome research currently limit the applicability of the top-down approach to large-scale discovery analyses. Nonetheless, "native mass spectrometry" experiments in which biological analytes are ionized by electrospray from nondenaturing solvents to preserve noncovalent interactions in the gas phase, have been used to analyze specific macromolecular assemblies including protein-protein and protein-ligand complexes (Hernández and Robinson 2007; Zhou et al. 2011; Leney and Heck 2017).

4.3 Bottom-Up (Shotgun) Proteomics

Instead of top-down intact protein analysis, "bottom-up" proteomics has been the more practical approach and has been widely adapted in the field (Aebersold and Mann 2016). In the bottom-up strategy, peptides are generated by enzymatic digestion of proteins with sequence-specific, exogenous proteases such as trypsin. The resulting peptides are separated by reversed-phase liquid chromatography (LC) and injected into the hyphenated tandem mass spectrometer. Peptides are isolated in the gas phase and subjected to fragmentation, thereby generating tandem mass spectra (MS/MS; MS^2). Collision-induced dissociation (CID) and higher-energy collisional dissociation (HCD) are two of the most commonly used fragmentation techniques to generate sequence information for peptide identification. Electron-transfer dissociation (ETD) and electron-capture dissociation (ECD) can be useful alternative strategies for the identification of larger and post-translationally modified peptides. Post-translational modifications (PTMs) such as phosphorylation and glycosylation are labile and readily lost over peptide backbone fragmentation (Mikesh et al. 2006; Syka et al. 2004; Zubarev et al. 2000). The resulting MS/MS fragmentation data are submitted to database search engines (i.e., MASCOT (Perkins et al. 1999), SEQUEST (Eng et al. 1994), X!Tandem (Craig and Beavis 2004), MyriMatch (Tabb et al. 2007), and OMSSA (Geer et al. 2004)) for protein/peptide identification. These search engines match and score the empirically acquired spectra against theoretically predicted fragmentation patterns of peptides derived from in silico digestions of proteins stored in protein sequence databases (Nesvizhskii 2010; Eng et al. 2011). Alternatively, MS/MS spectra can be matched via correlation analysis to previously observed and identified spectra using spectral library search engines such as SpectraST (Lam et al. 2007), X!Hunter (Craig et al. 2006), and BiblioSpec (Frewen et al. 2006). Though spectral library searching is typically considered to be

a more sensitive approach than sequence database searches, its adaption in the field has been fairly limited (Deutsch et al. 2018). PeptideAtlas (Desiere et al. 2004), the Global Proteome Machine Database (Craig et al. 2006), and the MassIVE Knowledge Base (Wang et al. 2018) are efforts to leverage the large number of peptide identifications contained in public proteomics datasets to create spectral library resources that can support future proteomics experiments.

Peptide sequences can also be derived from MS/MS fragmentation data by de novo sequencing approaches using algorithms including PEAKS (Ma et al. 2003), PepNovo (Frank and Pevzner 2005), Novor (Ma 2015), and Lutefisk (Taylor and Johnson 2001) that do not rely on reference databases (Allmer 2011). De novo sequencing frameworks designed for top-down proteomics can be advantageous in the analysis of high-resolution bottom-up MS/MS datasets (Vyatkina et al. 2017).

Combining the results of multiple search engines with tools such as iProphet (Shteynberg et al. 2011) can improve the confidence of peptide-spectrum matches and increase the overall number of distinct peptides and proteins identified since each search engine has its own specific strengths which can be complementary to others (Shteynberg et al. 2013).

Currently, there are two major data acquisition strategies used in bottom-up proteomics: The preferred method for proteome discovery is data-dependent acquisition (DDA), which aims to maximize the number of protein and peptide identifications per experiment to achieve comprehensive proteome coverage. Hallmarks of this approach include the 1-h yeast proteome (Hebert et al. 2013b) and draft maps of the human proteome with coverages of up to 92% of the protein-coding sequences (Wilhelm et al. 2014; Kim et al. 2014). To achieve this level of proteome coverage, additional fractionation techniques (strong anion exchange; off-gel electrophoresis) were employed to distribute sample complexity across additional data acquisitions. Applied on single cell lines (i.e., HeLa human cervical carcinoma), over 10,255 proteoforms stemming from 9205 genes can be identified by deep proteomics analysis (Nagaraj et al. 2011). Proteomics analyses of a panel of 11 commonly studied cell lines (Geiger et al. 2012) and the NCI-60 panel of 59 cancer cell lines (Gholami et al. 2013) suggests that at least ~10,000 proteins are about the average proteome coverage of a human cell line. A more recent study showed that adding an off-line high pH peptide fractionation step prior to low pH LC-MS/MS analysis can deepen the protein coverage even further to over 12,000 proteins for HeLa cells (Bekker-Jensen et al. 2017). A key strength of the described DDA methods is the fact that no a priori knowledge about the identity of the expected proteins is required and therefore unanticipated proteins and PTMs can be discovered, potentially providing new biological understanding.

Data-independent acquisition (DIA) also referred to as SWATH (Sequential Windowed data-independent Acquisition of Total High-resolution) is a more recently developed methodology that aims to obtain complete fragment ion coverage across samples (Ludwig et al. 2018). In DDA experiments, a full precursor ion spectrum of all co-eluting peptides is acquired at the MS^1 level, after which as many as possible precursor peptides are isolated, fragmented, and MS^2 spectra acquired within the instrument cycle time. In DIA experiments by contrast, predetermined

windows of m/z values are sequentially isolated for fragmentation (Gillet et al. 2012). In each instrument cycle, the entire precursor ion m/z range gets fragmented, resulting in highly multiplexed fragment ion spectra. Precursor-fragment ion relationships can be reconstructed with bioinformatic tools such as DIA-Umpire (Tsou et al. 2015, 2016) to create "pseudo"-spectra that are conventionally searched against protein databases to create internal spectral libraries that contain peptide identifications. These internal spectral libraries or external spectral libraries built from DDA data are then used to perform targeted extraction (Röst et al. 2014). Key advantage of the DIA approach is its unbiased nature: All precursor and all fragment ions are acquired all the time without losing low abundant ions; the identities of quantified peptides do not need to be specified a priori, which is ideal when the data is acquired over the course of a multi-year study. DIA measurements comprise an archival record of the sample content that can be re-interrogated when new proteins, proteoforms, or post-translational modifications sites of interest emerge.

4.4 Relative Quantitation in Bottom-Up Proteomics

In bottom-up proteomics, quantitation is achieved by either label-free or stable isotope labeling methods (Bantscheff et al. 2012). Stable isotope-based methods are the gold standard for quantification; however they require metabolic labeling or an additional chemical labeling step during sample preparation. Label-free approaches are simpler and more economical, providing relative quantitation for an unlimited number of samples (including clinical specimens) and can be based on either DDA or DIA datasets (Nahnsen et al. 2013). State-of-the-art mass spectrometers provide the necessary high mass resolution and high mass accuracy that are required for the accurate extraction of ion chromatograms (XICs; elution profiles) of precursor ions at the MS^1 level that are used to determine peptide quantities. In the past, when bottom-up proteomics was mostly performed on low-resolution ion trap instruments, the number of identified MS/MS spectra for a given peptide (spectral counts) was used as a surrogate measurement for peptide abundance (Ishihama et al. 2005). While the spectral count approach has been used to create one of the drafts of the human proteome (Kim et al. 2014), XIC-based approaches are now the most commonly employed label-free methodology due to their superior sensitivity. By aligning the retention times of XIC areas and propagating MS/MS-based peptide identifications across data acquisitions ("matching between runs"), the overall number of detectable peptides between samples can be boosted which leads to more comprehensive comparative analyses (Bateman et al. 2013). Numerous academic and commercial proteomics data analysis packages including PEAKS (Ma et al. 2003) and Scaffold (Searle 2010) offer label-free quantitative workflows in addition to their identification pipelines (Nahnsen et al. 2013; Mueller et al. 2008). Particularly noteworthy is the continuously expanding proteomics software tool suite under the MaxQuant umbrella which is freely available and has become one of the most widely used proteomics data analysis platforms. MaxQuant incorporates the peptide database search engine Andromeda (Cox et al. 2011) and the MaxLFQ

workflow for label-free quantitation (Cox et al. 2014) and supports as well other MS^1- and MS^2-level (isobaric) labeling approaches (Tyanova et al. 2016).

In contrast to the stochastic precursor ion selection in DDA, DIA systemically parallelizes the fragmentation of all detectable ions, thereby minimizing selection bias, which in turn results in improved dynamic range and sensitivity. Specific peptides can be identified and quantified by applying targeted extraction of either MS^1 precursor or MS^2 fragment ion intensities using spectral library-based OpenSWATH (Röst et al. 2014), Skyline (Maclean et al. 2010), or commercial software (PeakView SWATH 2.0, SCIEX; Spectronaut, Biognosys). The performance of these "peptide-centric" query tools in terms of identification precision, robustness, and specificity has been benchmarked against reference datasets and compared to the "data-centric" DIA-Umpire approach (Tsou et al. 2015) that does not rely on existing assay libraries (Navarro et al. 2016). Targeted extraction relies on the generation of sample-specific assay libraries that contain precursor and fragment ion m/z values, normalized retention times, and relative ion intensities of targeted peptides. Retention times are typically normalized using a set of reference peptides (Escher et al. 2012). DIA studies often rely on sample-specific libraries that are acquired on the same instrument in DDA mode prior to the DIA analysis (Gillet et al. 2012; Röst et al. 2014; Hüttenhain et al. 2013). Alternatively, repositories of assay libraries for human proteins have been created that are optimized for specific MS instruments. These resources contribute to simplified and reproducible targeted SWATH/DIA analysis across laboratories (Rosenberger et al. 2014). A multi-laboratory evaluation study across 11 sites demonstrated that SWATH acquisitions are capable of reproducibly detecting and quantifying a large-scale protein set (Collins et al. 2017).

4.5 Multiplexed Quantitation Using Stable Isotope Labeling Methods

The analysis of cancer signaling networks requires the ability to quantify proteins across multiple conditions so that temporal dynamics can be captured. A broad variety of chemical and metabolic stable isotope labeling methods have been developed that allow for multiplexing (Gevaert et al. 2008). Stable isotope labeling strategies can provide relative and absolute quantitation; however, the specifics of the labeling reactions can limit the number of samples that can be interrogated in contrast to label-free approaches. Isotope-coded affinity tags (ICAT) are one of the first stable isotope chemical labeling reagents that became widely adapted in proteomics (Gygi et al. 1999a). ICAT reagents are comprised of a reactive group specific toward cysteinyl residues, a stable isotope label (heavy/light), and a biotin affinity tag for selective enrichment to reduce sample complexity. ICAT allows for the duplex analysis for comparison of protein levels across two biological states. The exclusive reliance of ICAT on cysteine-containing peptides limits its general applicability as quantitation approach, and it has been mostly replaced by a new generation of isobaric labeling strategies based on *N*-hydroxysuccinimide (NHS) chemistry. The TMT (tandem mass tag) (Thompson et al. 2003) and iTRAQ (isobaric tags for relative and absolute

quantitation) (Ross et al. 2004) labels share isobaric stable isotope moieties as design features, which render differentially labeled samples "silent" – indistinguishable during chromatographic separation and in precursor MS^1 acquisition. Only upon MS/MS fragmentation the low molecular weight reporter ions are released, and their relative ion abundances are used for quantitation. Currently, there are up to eight reporter ions available for iTRAQ (Choe et al. 2007) and up to ten for TMT (Erickson et al. 2017), each allowing for multiplexed analysis in single LC-MS/MS experiments. For projects entailing larger sample numbers, one of the isotope channels is typically used for a control reference mixture.

The dynamic range of isobaric multiplex quantitation methodologies can be limited by isotopic contamination, background interference, low signal-to-noise ratio, and ratio compression (Ow et al. 2009; Karp et al. 2010). Applying an additional isolation and fragmentation event (MS^3 scan) (Ting et al. 2011) and gas-phase purification through proton transfer ion-ion reactions (Wenger et al. 2011) has been shown to eliminate interferences. Co-isolating and co-fragmenting of multiple MS^2 fragments (MultiNotch MS^3) can boost sensitivity and improve the dynamic range of the isobaric tagging approach (Mcalister et al. 2014).

Dimethyl labeling using different isotopomers of formaldehyde provides a more economical triplex stable isotope quantitation method at the peptide level (Boersema et al. 2008). Chemical isotope labels are typically introduced late in the sample preparation process, which makes these labeling strategies broadly applicable; however, at the same time, they are more susceptible to variability introduced during processing.

SILAC (stable isotope labeling by amino acids) is a metabolic labeling method alternative to chemical isotope tags (Mann 2006). SILAC relies on the in vitro incorporation of essential amino acids that feature substituted stable isotope nuclei (e.g., Arg or Lys labeled with ^{13}C or ^{15}N). SILAC labeling is insensitive to variability introduced at the sample processing and analysis stage since all sample handling issues affect all proteins and peptides equally. SILAC and ^{15}N metabolic labeling has been used for comparative proteomics analysis in cell culture systems (Ong et al. 2002; Everley et al. 2004, 2006) and model organisms including yeast (de Godoy et al. 2008), *C. elegans* and *D. melanogaster* (Sury et al. 2010), and rodents (Kruger et al. 2008; Wu et al. 2004). Full incorporation into the entire organisms requires feeding more than one generation exclusively with the essential, stable isotopically labeled lysine amino acids. A comprehensive analysis employing triple SILAC-based proteomics (using Arg0, Lys0; Arg6-L-$^{13}C_6$ and Lys4-L-$^{2}H_4$; Arg10-L-$^{13}C_6{}^{15}N_4$ and Lys8-L-$^{13}C_6{}^{15}N_2$), RNA-seq-based transcriptomic profiling, and antibody-based confocal microscopy revealed that three functionally different human cancer cell lines shared expression levels for more than half of their expressed genes, while close to 20% were substantially altered (Lundberg et al. 2010).

In the super-SILAC method, lysates from multiple SILAC-labeled cancer cell lines are combined to serve as internal, isotopically labeled peptide standards to measure fold change ratios between human tumor proteomes (Geiger et al. 2010). By combining SILAC and TMT labeling in the same experiment, a strategy termed

"hyperplexing," it is possible to extend the number of samples that can be quantified in the same LC-MS run (Dephoure and Gygi 2012).

The advent of mass spectrometers capable of ultra-high mass resolution (>200,000) made it possible to reveal the small mass differences (milliDaltons) introduced by the differences in the neuron-binding energetics of isotopes such as ^{2}H (+ 1.0062), ^{13}C (+ 1.0034), and ^{15}N (+ 0.997). The neuron encoding (NeuCode) method (available as amine-reactive labels and SILAC reagents) takes advantage of the ability to embed these mass defect-based neutron signatures into isotopologues. At standard resolution, these isotopologues are concealed during MS^{1} and MS/MS analysis and therefore do not increase spectral complexity (Hebert et al. 2013a). The multiplexed quantitative information is only revealed at high-resolution scans. NeuCode is applicable to DDA (Overmyer et al. 2018) and DIA approaches (Minogue et al. 2015) as well as targeted proteomics (Potts et al. 2016) and top-down applications (Rhoads et al. 2014; Shortreed et al. 2016).

4.6 Quantitation by Targeted Proteomics

Targeted proteomics provides accurate and quantitative measurements of protein abundances and thereby enables hypothesis-driven research using mass spectrometry (Picotti et al. 2013). In contrast to DDA- and DIA-based proteomics analyses, the identities of the proteins of interest are known a priori in targeted proteomics experiments. For any given protein, peptides are selected that are "proteotypic," meaning that each peptide has a unique sequence, is readily detected by MS, and has been repeatedly and consistently identified in previous studies (Mallick et al. 2007). By selectively subjecting these proteotypic peptides to precursor ion isolation and continuous fragmentation, characteristic fragment (product) ion abundances for the most intense transitions can be recorded over the chromatographic elution profile, and this information is then used to estimate relative protein abundances. These types of experiments are typically performed on triple quadrupole instruments operating in multiple reaction monitoring (MRM) mode, which is also referred to as selected reaction monitoring (SRM). To increase specificity, typically multiple product ions are measured. Absolute protein abundances can be determined by using spike-in, isotopically labeled reference peptides (Gerber et al. 2003) or mTRAQ chemically labeled standards (Desouza et al. 2008) or in label-free format when anchor proteins are used to create a quantitation model (Ludwig et al. 2011). An efficient method to define custom MRM assay conditions in high-throughput format is through the usage of crude synthetic peptide libraries (Picotti et al. 2010). To achieve proteome-wide coverage for absolute protein quantification, an in vitro protein expression system has been used to synthesize over 18,000 recombinant proteins from full-length human cDNA libraries, which were then digested and labeled with mTRAQ (Matsumoto et al. 2017). Alternatively, ProteomeTools is a brute force project to create a resource comprised of the

comprehensive LC-MS analysis of over 1.4 synthetic million peptides that cover tryptic and non-tryptic peptides representative of the canonical human proteome, as well as additional peptides covering splicing variants, post-translational modifications, and other sequences representing interesting biology such as disease-associated mutations (Zolg et al. 2017).

Compared to shotgun proteomics approaches, MRM assays provide higher sensitivity, specificity, and a broad dynamic range. Once established, individual MRM assays can be multiplexed at the peptide level (Picotti and Aebersold 2012). Measurements have been shown to be highly reproducible across laboratory sites (Addona et al. 2009). SRMAtlas (www.srmatlas.org) and PASSEL (www.peptideatlas.org/passel) both host freely accessible proteome-wide assay libraries along with empirical performance data that facilitate the design of targeted MRM assays (Farrah et al. 2012; Kusebauch et al. 2014, 2016). MRM assays for 1157 cancer-associated proteins have been developed, of which 182 were detected in depleted plasma and 408 in urine across a cohort of cancer patients and healthy controls using a label-free MRM strategy (Hüttenhain et al. 2012).

By combining peptide immunoaffinity enrichment with stable isotope-labeled standards and MRM-MS, it is possible to create automated, multiplexed assays with sufficient sensitivity to quantify low-abundance target proteins in plasma as an alternative to traditional enzyme-linked immunosorbent assay (ELISA)-based testing (Whiteaker et al. 2010).

The advent of high-resolution/accurate mass (HRAM) instrumentation has enabled the development of the parallel reaction monitoring method (PRM), in which the monitoring of a single product ion in an MRM assay is substituted with the parallel detection of all target product ions in a high-resolution MS/MS analysis (Peterson et al. 2012; Bourmaud et al. 2016). While MRM and PRM provide the best quantitation performance, both are throughput limited in terms of how many proteins can be quantified in a single MS experiment. SWATH/DIA provides a compelling alternative for reproducible quantitation in which a targeted data analysis strategy is employed to extract specific fragment ion abundances out of the comprehensive fragment ion map provided by the DIA dataset. Similar to MRM/PRM, reference libraries containing SWATH assay conditions can be built (Schubert et al. 2015) and shared via repositories (Rosenberger et al. 2014). SWATH/DIA assays have been shown to perform well across multiple laboratory sites (Collins et al. 2017). Additional throughput can be achieved for targeted proteomics assays when multiplexing is extended to the sample level by utilizing isobaric labels. In the TOMAHAQ method, synthetic TMT0-labeled spiked-in peptides trigger the MultiNotch MS^3 acquisition of co-eluting TMT10-labeled endogenous peptides, which allowed for the quantitation of 69 target proteins across 180 cancer cells within 48 h (Erickson et al. 2017). The setup and data analysis for this approach have been simplified by the recent development of the TomahaqCompanion tool (Rose et al. 2018).

By carefully selecting protein targets based on their involvement in particular biochemical pathways, it is possible to quantitatively investigate the response of cellular systems to external stimulation (Matsumoto and Nakayama 2018). The

multiplex MRM approach has been used to study the protein expression in major metabolic energy pathways of breast cancer cells in response to hypoxia, glucose deprivation, and estradiol stimulation (Drabovich et al. 2012; Murphy and Pinto 2010). Leveraging their in vitro proteome-assisted MRM assay library (iMPAQT) that covers over 18,000 proteins, Matsumoto et al. were able to explore the global impact of oncogenic transformation on fibroblasts (2017). Alternatively, by integrating detailed information about biological processes on the basis of literature evidence and computational predictions, it is possible to carefully select protein quantitation targets that can serve as sentinels or proxies for system responses (Soste et al. 2014).

4.7 Characterization of Post-translational Modifications

With continued improvements in mass accuracy, resolution, and sensitivity of mass spectrometry instruments, proteomic expression analyses feature deeper proteome and higher protein sequence coverages that enable more exhaustive characterizations of post-translational modifications (PTMs). PTMs including phosphorylation, glycosylation, and ubiquitination are important modulators of protein function: For example, most proteolytic enzymes are activated from their inactive precursor (zymogen) state by proteolytic cleavage (Klein et al. 2017). Many phosphorylations lead to protein conformational changes that modulate protein activity, i.e., protein binding. Ubiquitination marks proteins for degradation. Glycosylation often regulates protein function and enzymatic activities, alters protein-protein interactions, and changes the subcellular localization of numerous proteins. In mass spectrometric analyses, most PTMs lead to characteristic mass shifts in MS^1 spectra, and their location on specific amino acid residues can be determined by fragmentation analysis. However, the combinatorial nature of post-translational modifications creates a heterogeneity that constitutes a formidable analytical challenge as the vast structural diversity that can be generated via oligomerization and branching of glycans (complex carbohydrates) illustrates (Laine 1994). Hundreds of protein modification kinds (biological and artificial) have been reported in the Unimod (Creasy and Cottrell 2004) and RESID (Garavelli 2004) databases. The most actively studied post-translational modifications include phosphorylation, methylation, ubiquitination, methylation, acetylation, and O-GlcNAcylation (Doll and Burlingame 2015). Together, over 260,000 PTM sites have been identified in the human proteome so far (Doll and Burlingame 2015). Comprehensive information on empirically observed in vivo and in vitro post-translational modifications can be found in online bioinformatic resources including PhosphoSitePlus (PSP) (www.phosphosite.org), iPTMnet (https://research.bioinformatics.udel.edu/iptmnet/), and Phospho.ELM (http://phospho.elm.eu.org/) along with additional tools useful for PTM analysis (Hornbeck et al. 2012; Huang et al. 2018; Dinkel et al. 2011).

4.8 Phosphorylation

Protein phosphorylation is one of the central means by which cells transiently modulate protein function as exemplified by signal transduction pathways. The localization, the extent of phosphorylation, and the site-specific occupancy or stoichiometry are important determinants of protein functional modulation. Phosphorylation states are mediated by a network of kinases that phosphorylate serine, threonine, and tyrosine residues and phosphatases that remove phosphorylations. Deregulated kinase activities have been associated with the ability of cancer cells to circumvent physiological constraints on cell proliferation. Kinase inhibition (i.e., of the serine/threonine kinase mammalian target of rapamycin (mTOR)) has emerged as one of the most heavily pursued classes of drug targets in oncology (Dowling et al. 2010). With over 518 genes identified, protein kinases are one of the largest protein families in eukaryotes (Manning et al. 2002). It is estimated that a typical eukaryotic cell harbors between 700,000 and 1000,000 potential phosphorylation sites (Ubersax and Ferrell 2007; Boersema et al. 2010). Analysis of 50,000 phosphopeptides in HeLa S3 cancer cells revealed that at least three-quarters of the 11,000 identified proteins were phosphorylated (Sharma et al. 2014). Interestingly, the 150 most abundant phosphopeptides accounted for 20% of the cumulative phosphopeptide signal (Sharma et al. 2014). Phosphoproteomics analysis of nine mouse tissues (12,000 proteins; ~36,000 phosphorylation sites) revealed that most phosphoproteins are widely expressed but display tissue-specific phosphorylation to adapt to tissue function (Huttlin et al. 2010).

Phosphotyrosine accounts for only 1% of phosphorylations, owing to its primary regulatory and not structural role in proteins and a short half-life due to the presence of highly active phosphotyrosine phosphatases (Sharma et al. 2014). Many phosphoproteins such as transcription factors and protein kinases have low copy numbers. Combined with the substoichiometric levels observed for many regulatory protein phosphorylations, enrichment strategies are necessary to comprehensively profile protein phosphorylations (Macek et al. 2009). Enrichment can be performed at the phosphoprotein level prior to digestion using immobilized metal affinity chromatography (IMAC) (Collins et al. 2005) or after digestion using phosphopeptide enrichment by metal oxide affinity chromatography (e.g., using titanium dioxide (TiO2)) or IMAC. In the case of phosphotyrosine, immunoaffinity purification using phosphotyrosine-specific antibodies is preferred (Boersema et al. 2010; Kettenbach and Gerber 2011; Rush et al. 2005; Breitkopf and Asara 2012). Mass spectrometric characterization of phosphopeptides is challenging due to their overall low abundance, susceptibility to ion suppression, and limited fragmentation patterns (Dreier et al. 2018). Phosphopeptide-selective mass spectrometric detection methods include precursor ion and neutral loss scanning based on the diagnostic PO_3^- and H_3PO_4 ion losses that are caused by the lability of the O-phosphate bond in collision-induced dissociation (Le Blanc et al. 2003; Carr et al. 2005). Compared to pSer and pThr, phosphorylations of tyrosine (pTyr) are relatively stable and remain attached to MS/MS fragments, which facilitates their analysis. Also, pTyr yields characteristic immonium ions that can be used as an alternative means to

identify phosphorylation sites (Steen et al. 2003). In ion trap instruments, detection of neutral losses can be used to trigger the acquisition of MS^3 spectra in which the neutral loss precursor ion undergoes an additional round of isolation and fragmentation to yield better fragmentation coverage (Gruhler et al. 2005). Peptide fragmentation by ETD or ECD yields more extensive peptide backbone cleavages without shedding the labile phosphate groups first which, in turn, also facilitates phosphopeptide identification (Chi et al. 2007; Stensballe et al. 2000). Large-scale, quantitative phosphoproteomics has been used to define the downstream signaling networks of mTOR, identifying Grb10 as a potential mTORC1-regulated tumor suppressor (Hsu et al. 2011; Yu et al. 2011). The dynamic nature of the phosphoproteome mandates the acquisition of temporal profiles of the in vivo phosphoproteome to capture the cellular response upon stimulation (Olsen et al. 2006). By streamlining conventional multi-step phosphoproteomics workflows into a simplified parallel 96-well plate format protocol, sufficient sample throughput is now achievable to perform global profiling of phosphorylation in a time-resolved fashion (Humphrey et al. 2015). The NCI Clinical Proteomics Tumor Analysis Consortium (CPTAC) recently provided an optimized, highly reproducible workflow for proteome/phosphoproteome analysis that utilizes TMT-10 for multiplexed quantitation of over 10,000 proteins in a breast cancer xenograft model (Mertins et al. 2018).

An inherent challenge in large-scale phosphoproteomics analyses is the fact that changes in phosphoprotein expression levels can interfere with the interpretation of site-specific phosphorylation stoichiometries (Wu et al. 2011). Measuring the degree of phosphorylation requires the quantification of the cognate phosphorylated and non-phosphorylated peptides. This can be accomplished by splitting samples into two and forcing dephosphorylation in one fraction by phosphatase treatment and leaving the other fraction untreated. After differential stable isotope labeling, the two fractions are combined, and the degree of phosphorylation can be estimated by comparing the intensities of the differentially labeled unphosphorylated peptides (Zhang et al. 2002; Hegeman et al. 2004). Alternatively, spike-ins of synthetic isotopologues of the phosphorylated/non-phosphorylated peptides in conjunction with targeted mass spectrometry (MRM or PRM) can be used for absolute quantification of site-specific phosphorylation stoichiometry (Dekker et al. 2018; Jin et al. 2010). By normalizing for total phosphoprotein amount using multiple unmodified peptides, it is possible to estimate the degree of phosphorylation by calculating the ratios of phosphorylated/unphosphorylated peptide intensities for phosphoproteins of interest without stable isotope labeling (Steen et al. 2005). For large-scale phosphoproteomics studies that rely on phosphopeptide enrichment, parallel proteomics analyses can provide the necessary information on total phosphoprotein abundances to determine phosphorylation site stoichiometries (Wu et al. 2011; Olsen et al. 2010). Typical signaling pathway analysis is performed by collapsing discrete site measurements to the protein level. The curated PTMsigDB database aims to leverage site-specific post-translational modification information to capture signaling events more accurately as demonstrated in the phosphoproteome analysis of PI3K-inhibited breast cancer cells (Krug et al. 2018).

4.9 Ubiquitination

The ubiquitin-proteasome pathway controls the degradation of 80–90% of intracellular proteins. Ubiquitination is a process by which one or multiple ubiquitin monomers are covalently attached to the amino group at the protein N-terminus or at lysine side chains of substrate proteins, thereby forming branched proteins. Eukaryotic ubiquitin consists of 76 amino acids and is evolutionary conserved. Ubiquitination is catalyzed by a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3), which confers substrate specificity. De-ubiquitinating enzyme can reverse the ubiquitin conjugation, creating a steady state with poly-ubiquitinated proteins ($n > 4$) targeted for degradation by the 26S proteasome. As an important regulator of cell proliferation, differentiation, and survival, alterations of the ubiquitin ligase pathways have been linked to cancer (Ding et al. 2014; Mani and Gelmann 2005). Characterization of ubiquitination sites by mass spectrometry is commonly performed after antibody enrichment of peptides containing the Lys-GlyGly sequence that is formed during tryptic digestions of ubiquitinated proteins (Xu et al. 2010). More recently, an immunoaffinity strategy based on the recognition of the C-terminal 13 amino acids of ubiquitin has allowed for the identification of over 63,000 unique ubiquitination sites, including N-terminal ubiquitination, across 9200 proteins in 2 human cell lines (Akimov et al. 2018).

4.10 Proteogenomics

In an effort to elucidate how somatic gene mutations impact the cancer proteome and the post-translational modification landscape, CPTAC used quantitative MS and phosphoproteomics to characterize hundreds of ovarian, breast, and colon/rectal tumors whose genome and transcriptome were previously defined by The Cancer Genome Atlas (TCGA) (Mertins et al. 2016; Zhang et al. 2014, 2016). Integrating genomic and proteomics/phosphoproteomics measurements allowed to explore the effect of copy number alterations on protein abundance and test whether transcriptome-derived subtypes are reflected in protein expression patterns. Proteogenomics promises to deepen our understanding of cancer biology and identify alterations in cancer signaling pathways and potential therapeutic targets with higher levels of confidence. The human cancer proteome variation cancer database (CanProVar) provides a bridge between genomic and proteomics data by compiling protein sequence alterations in different types of cancers (Zhang et al. 2017; Li et al. 2010) along with extensive annotation, which can be used for the detection of variant peptides in shotgun and targeted proteomics experiments (Li et al. 2011).

4.11 Ultrasensitive Proteomics via Cellular Pre-fractionation

Given the microheterogeneity of the cancer microenvironment, it can be of advantage to analyze specific cell types individually in order to more accurately reveal their biochemical potentials. Cellular populations can be specifically purified by antibody-based methods such as fluorescence-activated cell sorting, CyTOF mass cytometry, or immune magnetic separation. CyTOF mass cytometry uses rare earth metals as unique antibody reporters that are monitored by inductively coupled plasma mass spectrometry (ICP-MS) in multiplex format to reveal marker expression in individual cells (Bandura et al. 2009). ICP-MS offers an extraordinary level of sensitivity which enables the detection of metal-labeled antibodies at levels corresponding to single cells. Alternatively, cellular subpopulations can be dissected from tissue using laser capture microdissection (LCM) prior to MS-based proteomics analysis (Altelaar and Heck 2012). In-depth LC-MS analysis of approximately 3000 LCM-derived tumor cells can yield the identification of 1000–2000 proteins (Umar et al. 2007; Wiśniewski et al. 2011), a number that can be boosted to over 4000 protein identifications from microdissected cells from formalin-fixed and paraffin-embedded human tissue specimens with the incorporation of additional off-line fractionation steps (Wiśniewski et al. 2011).

4.12 Imaging Mass Spectrometry

MALDI and secondary ion mass spectrometry (SIMS) imaging mass spectrometry (IMS) combine the parallel molecular detection by mass spectrometry with microscopic imaging to visualize the spatial distribution of proteins and metabolites (Cornett et al. 2007; Schwamborn and Caprioli 2010). MALDI-IMS yields 2D molecular maps that provide the localization and relative abundance of thousands of analytes in thin tissue sections with typical pixel size in the range of 50–200 μm in an untargeted manner (McDonnell and Heeren 2007; Schober et al. 2012). The discovery nature of MALDI imaging can be complemented by imaging mass cytometry, which utilizes the multiplexing capability of CyTOF mass cytometry for the targeted multiplexed localization of up to 32 proteins with subcellular resolution. This approach was pioneered to characterize tumor cell subpopulations and highlight the heterogeneity of human breast cancer microenvironments (Giesen et al. 2014).

4.13 Outlook

The field of mass spectrometry-based proteomics continues to rapidly evolve and mature. Each new generation of mass spectrometers pushes the limits of performance in terms of resolving power, mass accuracy, and sensitivity. Many of these improvements continue to trickle down into mainstream instrumentation available to the average user. How do these technological innovations impact the field?

Ultra-high resolution opens the window to investigate the fine structure of isotopologues. This advancement has already led to the development of novel stable isotope labeling strategies that take advantage of mass defect-based neutron encoding for multiplexed quantitation (Hebert et al. 2013a). The resolved isotopologues structures could also be harnessed by a next generation of informatic pipelines that capitalize on the encoded elemental composition information in an effort to improve peptide/protein identification rates.

In terms of sensitivity, one promising approach entails a switch from serial to parallel accumulation of MS precursor and subsequent release and fragmentation based on ion mobility. The speed and sensitivity of MS/MS experiments can be increased by parallel accumulation and serial fragmentation (PASEF) that is employed on trapped ion mobility-mass spectrometry (TIMS)-mass spectrometers (Meier et al. 2015). Other opportunities exist to increase sensitivity by improving and better integrating sample preparation and data acquisition workflows (Specht and Slavov 2018). Increased sensitivity will open up the transformative potential of single-cell proteomics, in which the contribution of each cell type to complex microenvironments such as cancers can be determined.

Integration with other omics approaches and resolving the spatial distribution of proteins are key aspects to reveal protein function and elucidate their role in physiology and pathology. The Human Protein Atlas project (www.proteinatlas.org) is a pioneering resource to study spatial proteomics across the major tissues and organs of the human body (Uhlen et al. 2015) and at the subcellular level (Thul et al. 2017) based on immunohistochemistry and complemented by RNA sequencing and mass spectrometry. The Human Pathology Atlas companion extends this groundbreaking system-level analysis to the transcriptome of the 17 major cancer types (Uhlén et al. 2017).

Finally, live monitoring of data acquisition will provide opportunities to fine-tune workflows in real time so that qualitative and quantitative performance can be optimized. The MaxQuant.Live framework is a first example of how real-time monitoring can be used for on-the-fly recalibration of mass and retention times which increases the efficiency of LC-MS experiments (Wichmann et al. 2018). In the future, further integration of entire workflows from automated sample preparation, data measurements, and data analysis will make the development of adaptive and smart data acquisitions a reality.

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Apoptosis in Cancer Cell Signaling and Current Therapeutic Possibilities

5

Ajay R. Wagh and Kakoli Bose

Abstract

Apoptosis is programmed cell death, which sustains the equilibrium between survival and death in eukaryotic cells. It is a tightly regulated cell death program that aims at eliminating harmful, damaged, or unwanted cells. This wisely programmed cell death is central in the development of all multicellular organisms, which is highlighted by the prevalence of diseases associated with abnormal apoptosis. For example, defect in apoptosis is a hallmark of cancer, whereas excessive cell death occurs in several neurodegenerative disorders. The cell death signals are responsible for maintenance of the genomic integrity, while defective cell death may stimulate carcinogenesis. These signals are convoluted and are controlled at various points. Tumor cells survive by taking help of several different molecular mechanisms to inhibit apoptosis and acquire resistance to apoptotic agents, for example, by the expression of anti-apoptotic proteins such as Bcl-2 or by the downregulation or mutation of pro-apoptotic proteins such as BAX. This chapter includes recent developments in the field and reviews new evidences of the interconnection between apoptosis and cancer. Various molecules that can be regulated to facilitate apoptosis in myriad of cancers are also enlisted. Overall, the chapter discusses about the development of various treatments and approaches to combat cancer by targeting anti-apoptotic proteins belonging to Bcl-2 and IAP families.

Keywords

Apoptosis · bcl-2 family members · Cancer · Therapeutics

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5.1 Introduction

5.1.1 Apoptosis: A Programmed Cell Death

The average adult human body makes around 50–70 billion new cells every day by somatic cell division also called mitosis. Therefore, to maintain a constant number of cells in the body, they must also die a natural death. In humans, about a million of cells are made every second through mitosis, whereas a similar number is destroyed by the process of apoptosis – this is known as cellular homeostasis (Vaux and Korsmeyer 1999). The word "apoptosis" (Greek origin) is divided into two parts, *apo* (from) and *ptosis* (falling), that describes falling of leaves from a tree (Wong 2011). Including apoptosis, there are various types and subtypes of cell death. The primary ones are as follows:

1. *Necrosis*: Uncontrolled cell death. It is generally the outcome of cellular injury and/or infection. Phenotypically, the cells burst, emitting their contents into the surrounding tissue fluid; this stimulates inflammation, pain, and swelling.
2. *Apoptosis* (programmed cell death): It is a programmed, multistep pathway that leads to cell death for the normal functioning of cellular growth and tissue homeostasis. It is developed as an irreversible practice to proficiently remove nonfunctional and old cells (Letai 2008). There are stepwise events, such as enzyme activation, required for this (unlike necrosis). In apoptosis, genetic control is preserved till the end (Vo and Letai 2010).
3. *Autophagy:* It is a naturally occurring controlled mechanism by which a cell can dismantle its redundant and defective components in a systematic manner (Yu et al. 2004).

Generally, the morphological features of a dying cell decide the mode of cell death. Consequently, apoptosis has been designated as *type I* programmed cell death, autophagy as *type II*, and necrosis as a type of death that combines characteristics of both *type I* and *type II*. With the advent of new biochemical methods over the past few decades, a more conclusive categorization of cell death types has become possible. Cell death classifications have yielded categories which render to biochemical characteristics, morphological features, immunological facets, or functional references. Normally, cell cleaning process or apoptosis starts with breaking down of the genetic materials across the nuclear margins, followed by cell fragmentation and generation of cell debris, which in turn are consumed by phagocytes (Figs. 5.1 and 5.2). A series of biochemical events leading to apoptosis includes activation of proteases like caspases, cleavage of DNA and proteins, and exposure of phosphatidylserine on the surface of the cell. Along with elimination of unwanted cells and tissues, apoptosis also plays a major role in development of the embryo and neurologic pruning. Categorically, phases of apoptosis include the *initiation phase* and *execution phase* (Kroemer et al. 2007).

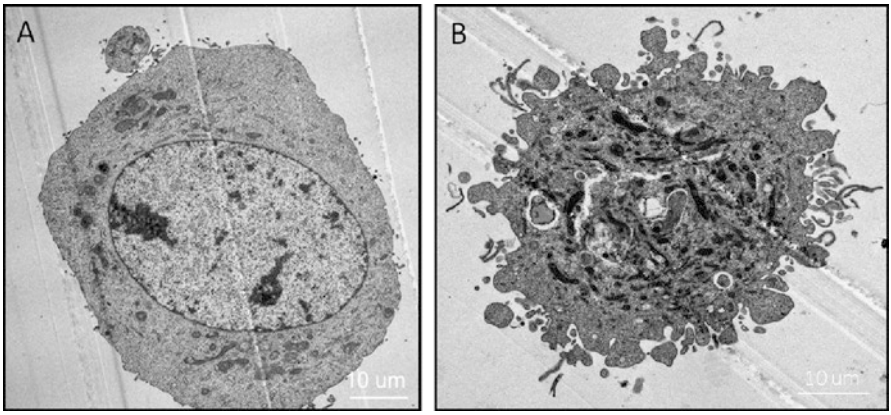

Fig. 5.1 Ultrastructural examination of typical apoptotic cell using transmission electron microscopy (TEM). Untreated control HEK293T cell. (**a**) exhibit normal cell morphology including normal cell nuclei, cytoplasm, and cell organelles. (**b**) Drug-treated cell indicates the appearance characteristics of apoptotic cell such as membrane blebbing, nuclear condensation, and damage to cell organelles. Magnification, x8,000. Image is under publication (*Bose Lab, unpublished results*)

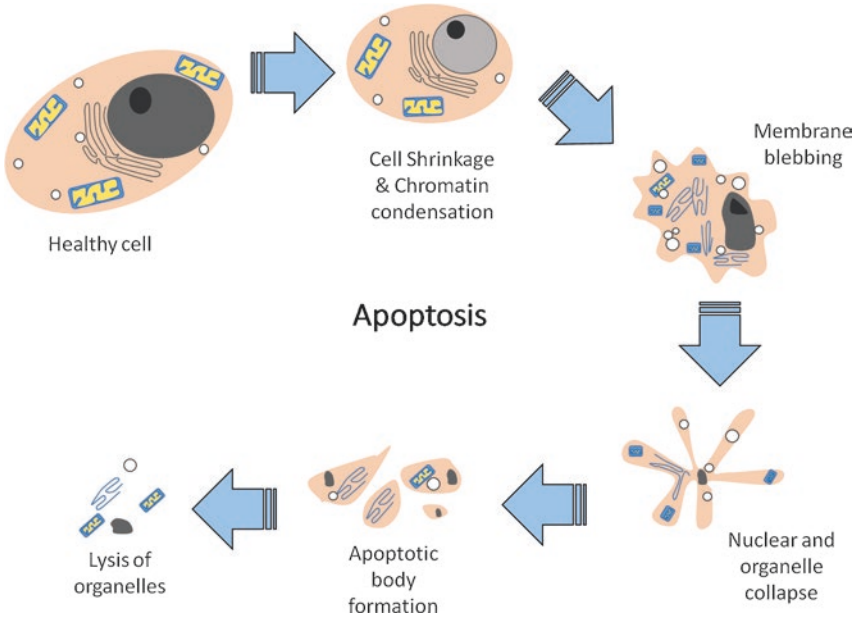

Fig. 5.2 Hallmarks of apoptotic cell death. Cartoon illustrating the basic stages of the apoptotic pathway. Apoptosis includes cellular shrinking, chromatin condensation, and margination at the nuclear periphery with the eventual formation of membrane-bound apoptotic bodies that contain organelles, cytosol, and nuclear fragments

5.1.2 Initiation Phase

This phase of apoptosis mostly involves a complex entanglement of a number of proteins. It depends on the cell type, apoptotic stimulus, and activation of proteases, nucleases, and other effector molecules. Normally, it gets activated by several intracellular and extracellular stresses (Bender and Martinou 2013). Extracellular signals that induce apoptosis include dysfunctioning growth factors, hypoxia, and environmental radiation. Intracellular signals include DNA damage, damage caused by chemotherapeutic drugs, telomere malfunction, and viral infections. The two different important pathways that mediate initiation phase are discussed in the following sections.

5.1.2.1 Extrinsic Pathway Mediated by Receptor

It is induced by external events of cells mediated by the death receptors. In this pathway, members of the tumor necrosis factor (TNF) receptor superfamily of transmembrane proteins are mainly involved. The most notable feature of TNF receptors is a region called "death domain" which roughly consists of 80 amino acids (Bradley and Pober 2001). Transferring death signals across cellular membrane is the most significant role of these death domain regions. Two most well-known death receptors are the type 1 TNF receptor (TNFR-1) and a related protein called Fas (CD95). Upon receiving apoptotic stimuli, receptors trimerize after binding to their respective extracellular ligands, TNF and Fas ligand (FasL) (Hengartner 2001). Post trimerization, these death receptors recruit adapter proteins such as TNF receptor-associated death domain (TRADD) and Fas-associated death domain (FADD) through homotypic protein-protein interactions. Subsequently, the adaptor molecules bind to initiator cysteine proteases such as caspase 8/10 through similar homotypic interactions and that forms a complex called death inducing signaling complex (DISC) (Singh et al. 2016). DISC formation is a prerequisite of autoactivation of upstream or initiator caspases 8/10 with the subsequent activation of downstream caspases 3/7 (Wong 2011) that lead to initiation of the caspase cascade, hence apoptosis (Schneider and Tschopp 2000). Extrinsic cell death pathway is strongly controlled by relative expression of both pro-apoptotic and anti-apoptotic proteins, and a disproportion in that regulation leads to several diseases.

Defects in Extrinsic Pathway Signaling and Carcinogenesis

Genetic studies on mice in which certain genes encode death ligands or their receptors have given sufficient proof of role in tumor suppression by cellular immune mechanisms. Fas ligand (FasL) is essential when there is cytotoxic T lymphocytes (CTL)-mediated killing of tumor cells, and TRAIL/Apo2L (TNF-related apoptosis-inducing ligands) is important for natural killer (NK)-cells-mediated tumor suppression. However, a number of cancer cells escape immune annihilation by resisting the response of the death receptor pathway to FasL. Although numerous available cancer cell lines express various cell surface receptors, they show an inherent resistance to TRAIL. It somehow indicates that during the course of evolution of tumors in vivo, an enforced selection of malignant clones takes place, which are

capable of defying immune attack. Some of the examples are downregulation of the Fas receptor, abundance of nonfunctioning or precursor of Fas receptors, and generation of large amount of soluble Fas receptor. Moreover, some cancer cells can execute a "counterattack" facilitated by Fas ligand that results in apoptotic reduction of activated tumor-sensitive lymphocytes (Koyama et al. 2001). Thus, it is very important to have a promising therapeutic approach that can rely on making extrinsic pathway to act on nonessential cells.

5.1.2.2 Intrinsic Pathway or In-House (Mitochondria Dependent)

This pathway is initiated by internal cell death gestures, mainly severe nuclear DNA damage, low oxygen content in the cells, viral infection, etc. It is majorly controlled by proteins in the Bcl-2 (B-cell lymphoma 2) family, comprising a total of 25 known proteins. They are involved to either excite (pro-apoptotic) or inhibit apoptosis (anti-apoptotic) (Hengartner 2000).Under normal conditions, there is an equilibrium among pro-apoptotic and anti-apoptotic proteins present in the cell**.**

The BH3-only (Bcl-2 Homology-3) proteins when respond to signs, such as loss of genetic material to undergo apoptosis, position themselves to the mitochondrial outer membrane and trigger the two important proteins Bax or Bak. When stimulated, Bax and Bak heterodimer cause mitochondrial outer membrane permeabilization (MOMP) (Bender and Martinou 2013). This heterodimer penetrates the membrane and creates pores all over the membrane that help other apoptotic factors like cytochrome-c to come out into the cytosol. Later on, it binds to caspase 3 forming a crucial compound/complex known as apoptosome comprising cytochrome-c, Apaf-1 (actin filament-associated protein 1), and nonfunctional initiator caspase-9 (Kroemer et al. 2007). The newly formed composite structure works by hydrolyzing adenosine triphosphate (ATP) and form a functional caspase 9. The active/functional caspase 9 further reacts with the executioner caspases 3, 6, and 7 to carry out their respective functions that ultimately help in cellular apoptosis. This pathway is distinct from the one emanating from extracellular signals. Apart from them, some apoptotic factors like Apoptosis-inducing factor (AIF), second mitochondria-derived activator of caspase (SMAC), and Omi/high-temperature requirement protein A-2 (HtrA2) are also exuded out from the intermembrane space of mitochondria into the cytoplasm (Kroemer et al. 2007).

Defects in Intrinsic Pathway Signaling and Carcinogenesis

One of the key proteins found to be associated with intrinsic pathway of apoptosis is caspase 9, whose impairment leads to detrimental consequences. For example, caspase-9-knockout mice show juvenile death due to abnormalities in the brain structure/function due to accumulation of increased number of unnecessary cells (Hakem et al. 1998; Kuida et al. 1998). Furthermore, lymphocytes within the thymus glands from the knockout mice display susceptibility toward common variety of anticancer drugs and chemo-radiations (Kuida et al. 1998).

There are reports suggesting reduced expression of caspase 9 in the epithelial cells from colorectal cancer patient's cells related to cells from healthy individual (Hector et al. 2012). Apart from that, anti-apoptotic proteins (e.g., Bcl-2, Bcl-X_L

(B-cell lymphoma 2-Xetra large), Bcl-2 L2 (B-cell lymphoma 2 like protein 2), and Mcl-1 (induced myeloid leukemia cell differentiation protein)), which have conserved BH1–BH4 domains are known to promote cell survival, whereas pro-apoptotic proteins are associated with stress-dependent apoptosis. There is a second group of multidomain BH3-only proteins such as Bax and Bak, which are essential for apoptosis (Reed 2006). Anti-apoptotic proteins block apoptosis by counteracting the actions of Bax/Bak complex through a known mechanism (Green 2006). Overexpression of anti-apoptotic Bcl-2 or Bcl-X_L occurs in more than half of all cancers (Amundson et al. 2000) by rendering cancer cells resistant to apoptotic stimuli that also include majority of cytotoxic anticancer drugs.

5.1.2.3 Perforin/Granzymes

As a part of human cellular immunity, in response to several stress conditions, cells such as cytotoxic T lymphocytes can cause apoptosis. The procedure involves activation of such cells followed by secretion of special proteins called perforin and its associated enzymes. Their main job is to form pores onto the cell membrane of the target cell. The additional particles use these pores to enter the cell. After entering the cell, they release their enzymes (granzymes A and B) that start execution of apoptosis by causing destruction of cellular structure and function (Trapani and Smyth 2002).

5.1.3 Execution Phase

The initiation phase activates the execution phase. The execution phase involves the activation of a group of cysteine proteases named as caspases which plays cell execution (Wong 2011; Bender and Martinou 2013). These proteins are ubiquitously present in all cell types, and their expression is enough to carry out its function. Among all, caspase 3 is a well-known executioner caspase. Once this protein takes charge as an executioner, apoptosis is definite. It is activated in major functions mainly DNA and chromatin damage, cell division, as well as signal transduction.

5.2 Resistance to Apoptosis in Cancer

Uncontrolled proliferation of cells give birth to variety of cancers (King and Cidlowski 1998). Moreover, the role of malfunctioning apoptotic machineries during cancer metastasis is well documented in myriad of cancers (Kerr and Searle 1972). Normally, tumor cells use various molecular mechanisms to acquire resistance to apoptosis. These processes mostly include upregulation of anti-apoptotic proteins like Bcl-2 and downregulation of pro-apoptotic proteins such as BAX. As regulation of both proteins is controlled/regulated by the p53 gene (Miyashita et al. 1994), some forms of B-cell lymphoma show Bcl-2 overexpression (Fig. 5.3). Therefore, many similar examples represent the first line of evidences that failure of apoptosis contributes to cancer (Vaux 1998).

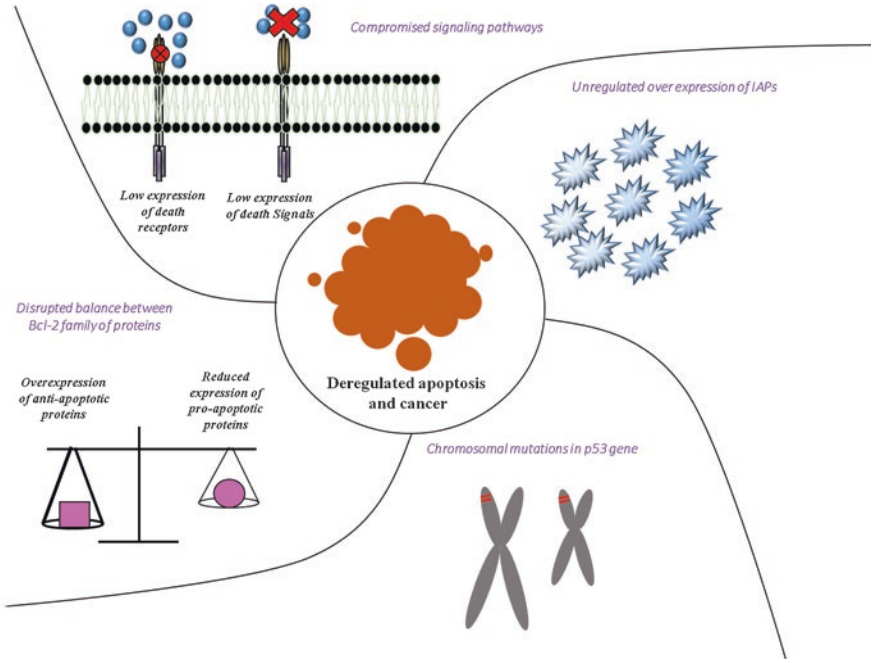

Fig. 5.3 Disrupted balance between Bcl-2 family of proteins: Many proteins have been reported to exert pro- or anti-apoptotic activity in the cell. It is not the absolute quantity but rather the ratio of these pro- and anti-apoptotic proteins that plays an important role in the regulation of cell death. Besides, over- and underexpression of certain genes have been found to contribute to carcinogenesis by reducing apoptosis in the cancer cells

5.3 Disrupted Balance of Pro-apoptotic and Anti-apoptotic Proteins

5.3.1 Role of Bcl-2 Family of Proteins in Apoptosis

This family of proteins is considered as a chief doorkeeper to the apoptotic response. Gene involving Bcl-2 family of proteins was first identified as one of the promoters of cell death whose functioning revolved around activation of pro-apoptotic proteins or inhibition of anti-apoptotic proteins. Anti- and pro-apoptotic members belonging to this group of structurally related proteins interact with each other. Among them, one of the subgroups include Bid, Bad, Bim, Bmf, Puma, and Noxa proteins that contain a single Bcl-2 homology 3 domain (BH3-only proteins) and have pro-apoptotic activity. Two other protein subcategories have multiple BH domains. The first subcategory, including Bcl-2-associated X protein (Bax), Bcl-2 homologous antagonist/killer (Bak), and Bcl-2 family apoptosis regulator (Bok), is pro-apoptotic; the other subcategory, including Bcl-2, Bcl-XL, and Mcl-1, is anti-apoptotic (Fig. 5.4). At least one BH domain is confined in each of the Bcl-2 family members and contributes to the functioning of the members (Strasser et al. 2011).

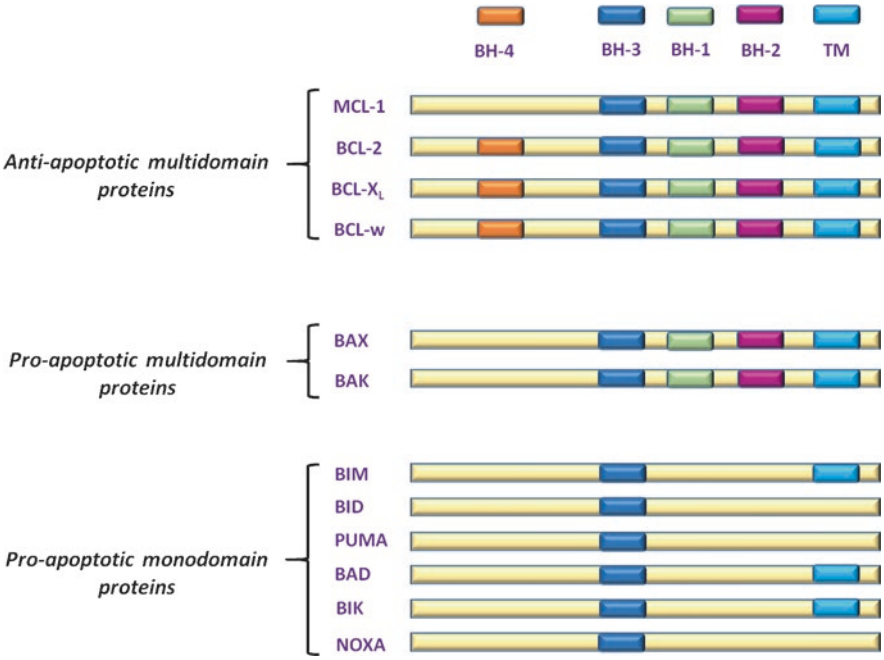

Fig. 5.4 Bcl-2 family: organization, functions, and characteristics. Schematic shows the important Bcl-2 family proteins in humans along with the main conserved structural motifs (BH domains), grouped according to function. TM, transmembrane domain. BH, Bcl-2 homology

The Bcl-2 family members can be classified into 3 functional groups: anti-apoptotic, pro-apoptotic *effectors*, and pro-apoptotic *activators*. According to a number of literature reports, pro-apoptotic activators, which comprise BH3 motifs, mostly function as important mediators in stress-related cellular responses like DNA damage (Certo et al. 2006). However, *effectors* group of Bcl-2 proteins initiates pore formation in the mitochondrial membrane after being activated by BH3 containing *activators* (Garcia-Saez 2012; Wei et al. 2001).

However, in preclinical models, it has been established that Bcl-2 binding to BH3-only *activators* disrupts the interactions between *activators* and pore-forming *effectors*, which in turn refrain the *effectors* from forming mitochondrial pores. The dynamic balance occurring between anti-apoptotic and pro-apoptotic members may involve combinations of under- or overexpression of one or more anti-apoptotic as well as pro-apoptotic proteins. This further helps to determine whether the cell initiates apoptosis or not (Strasser et al. 2011; Garcia-Saez 2012). For example, there are reports on Bcl-2 overexpression resulting survival of prostate tumor cells from apoptosis (Raffo et al. 1995). Moreover, in breast carcinoma and neuroblastoma cancer cells, overexpression of Bcl-2 is observed which leads to reduced TRAIL-induced apoptosis (Fulda et al. 2002). Similarly, various in vivo studies show that in **c**hronic lymphocytic leukemia (CLL) cells, Bcl-2/Bax ratio increases with the decrease in pro-apoptotic Bax protein. Moreover, it is also seen that apoptosis

induced by drug application decreases the ratio and rate of drug induction is inversely proportional to the ratio (Goolsby et al. 2005).

5.3.2 Tumor Cells May Become Dependent on Bcl-2 for Survival

Recently, a phenomenon known as "oncogene addiction" has been identified. It is the dependence of a cancer cell on one signaling pathway or hyperactive gene for the cell's survival and growth. Similar oncogene addiction is also seen in various cancer cells whose survival become directly dependent on pathways involving Bcl-2 proteins (Certo et al. 2006). In case of these tumor cells, the pro-apoptotic *activators* get sequestered due to Bcl-2 binding. However, with a sufficient increase in the *activators* quantity, Bcl-2 sequestering by those *activators* can be invoked resulting apoptosis. Hence, this sensitivity toward Bcl-2 modulation can be used as a therapeutic tool for the treatment of Bcl-2-dependent cancers (Deng et al. 2007).

5.4 Expression Levels in Various Cancers

Expressions of functional Bcl-2 (increased ratio of anti-apoptotic to pro-apoptotic proteins) alone can regulate the extent of developing cancer. Following are the different cancer types and its association with aberrant expression of Bcl-2 blood *cancer/leukemia.*

CLL patients have always shown a noticeable Bcl-2 gene rearrangement with upregulated expression (Hanada et al. 1993). Increased expression is also found frequently in **a**cute **m**yeloid **l**eukemia (AML) (Wei et al. 2001) and in almost all patients with **a**cute **l**ymphocytic **l**eukemia (ALL) (Gala et al. 1994).

5.4.1 Non-Hodgkin lymphoma

In follicular lymphoma, most of the patients shows a t(14;18) chromosomal translocation in abnormal cells (Tsujimoto et al. 1984), which is believed to cause overexpression of Bcl-2 protein (Chen-Levy et al. 1989). This is true with diffused large B-cell lymphoma patients, in whom relatively high Bcl-2 expression is found (Hermine et al. 1996).

5.4.2 Cancerous Solid Tumors

Involvement of Bcl-2 in non-hematologic cancers like small cell carcinoma, breast carcinoma, and prostate and lung cancers has been observed where its unregulated expressions lead to aforementioned malignancies (Karnak and Xu 2010; Hellemans et al. 1995; Jiang et al. 1995; Anagnostou et al. 2010). In case of lung cancer, overexpression in >85% of patients has been reported (Jiang et al. 1995). Moreover, in

Table 5.1 Clinical evidences in cancer

Malignancy	Bcl-2 expression levels
Chronic lymphocytic leukemia (CLL)	Relatively high levels are observed in more than 90% of CLL cases compared to normal peripheral blood lymphocytes (Hanada et al. 1993)
Acute myeloid leukemia (AML)	Reduced response to chemotherapy at high level of expression (Campos et al. 1993)
Acute lymphocytic leukemia (ALL)	High levels are found in nearly all patients with ALL (Gala et al. 1994).
Follicular lymphoma (FL)	Chromosomal translocation resulting in overexpression in nearly 90% of follicular B-cell lymphomas (Tsujimoto et al. 1984)
Diffuse large B-cell lymphoma (DLBCL)	Chromosomal translocations-induced Bcl-2 overexpression is also seen in approximately 20% of DLBCL (Huang et al. 2002). Overall survival rate within a particular subgroup of DLBCL is inversely proportional to Bcl-2 expression (Iqbal et al. 2006).
Cancerous solid tumors	Untamed Bcl-2 levels have been observed in various tumors like breast, prostate, and lung cancers (Karnak and Xu 2010; Hellemans et al. 1995; Jiang et al. 1995; Anagnostou et al. 2010).

neuroblastoma, colorectal, ovarian, and urinary bladder cancers, substantial levels of Bcl-2 expression have been observed (Henriksen et al. 1995; Lamers et al. 2012; Swellam et al. 2004; Zhao et al. 2005; Pena et al. 1999) (Table 5.1).

5.5 The p53 Protein

Tumor protein 53 (or TP 53) is a tumor-suppressor protein encoded by the *TP53* gene, due to its specific molecular weight, that is, 53 kDa. The association of p53 with cancer surfaced in the late 1990s, which showed that gain of its oncogenic function was the direct outcome of its mutation (Levine et al. 1991). p53 is widely known as the "guardian of the genome" due to its significant role in a large number of cellular processes like chromosomal segregation, cell cycle regulation, recombination of genetic material, gene amplification, as well as apoptosis (Oren and Rotter 1999; Lane 1992). More than 50% of different types of human cancers are associated with malfunctioning of p53 gene (Levine et al. 1991). Recently, abnormal expression of p53 has also been reported in melanoma cells where its reduced activity leads to melanoma cells proliferation (Avery-Kiejda et al. 2011) Similarly, one of the research groups observed decreased survival, reduced apoptosis, and enhanced level of proliferative markers in a mouse model expressing truncated version of p53(Δ122p53) (Slatter et al. 2011). Moreover, p53 knockdown also decreases the

growth of cellular colonies in malignant tumor cells, which is due to apoptotic induction (Vikhanskaya et al. 2007).

5.6 Inhibitors of Apoptosis Proteins (IAPs)

Regulation of apoptosis involves a group of structurally related and functionally analogous group of proteins called the IAPs. The family is characterized by having one or two Baculovirus IAP repeat (BIR) protein domain (LaCasse et al. 2008). Since then, eight IAPs have been discovered, namely, Apollon (BRUCE, BIRC6), c-IAP1 (BIRC2), c-IAP2 (BIRC3), IAP-like protein 2 (BIRC8), Livin/ML-IAP (BIRC7), NAIP (BIRC1), survivin (BIRC5), and X-linked IAP (XIAP, BIRC4) (Vucic and Fairbrother 2007). Generally, they are involved in inhibition of caspase activity by either degrading the active forms of caspases or holistically interacting with them to prevent substrate-caspase binding (Wei et al. 2008).

It has been reported that in many cancers, there is a versatile expression of IAP proteins. For example, unusual levels of IAP were identified in the pancreatic cancer cells due to its link with chemoresistance. A study has further reported that there is a significant increase in the levels of cIAP-2 in many cancers (Lopes et al. 2007). According to recent reports, in cancers such as melanoma and lymphoma, significantly high levels of Livin/ML-IAP (BIRC7) has been reported (Vucic et al. 2000; Ashhab et al. 2001).While in gliomas, Apollon (BRUCE, BIRC6) upregulation exhibited chemoresistance for drugs cisplatin and camptothecin (Chen et al. 1999). Overexpression of survivin in various cancers has also been described. Together with XIAP, overexpression of survivin has been observed in non-small-cell lung carcinomas (NSCLCs). According to their study, tumors that upregulate both the proteins are capable of chemoresistance in all the apoptosis-inducing conditions (Krepela et al. 2009).

5.7 Potential Treatment Strategies Against Cancer

Role of dysregulated apoptosis pathways not only is limited to initiation of malignancy and cancer development but also leads to chemo- and radiation-resistant tumor cells. Initial responses of the cancer cells toward different therapies subside gradually as the cells start resisting apoptosis by increasing anti-apoptotic protein expressions which in turn block the pro-apoptotic pathways. Therefore, modern cancer research and drug development predominantly involve in finding the fundamental mechanisms governing apoptosis which may help in constructing therapeutic strategies to combat with cancer resistance and enhance patient survival.

5.7.1 Bcl-2 Antagonists

Bcl-2 family of proteins can be therapeutically targeted by developing Bcl-2 antagonists which may inhibit their activities as well as silence the upregulated anti-apoptotic genes or proteins. Significant role of Bcl-2 proteins in disrupting apoptosis has led to several drug-based advances in twenty-first century, which focus mainly on developing molecules that antagonize Bcl-2 family of proteins and quantitatively reduce their levels in cells. For example, a drug has been developed on the basis of an antisense nucleotide to *bcl-2* and has been successfully tested in a wide range of human cancers (Frankel 2003). Moreover, Bcl-2 antagonists have been designed by a number of research groups to primarily mimic BH3-only peptides (Baell and Huang 2002; Kutzki et al. 2002; Becattini et al. 2004; Qian et al. 2004). These agents are useful in treating cancers such as follicular lymphoma in which elevated levels of Bcl-2 occur.

5.7.2 SMAC (Second Mitochondria-Derived Activator of Caspase)/Diablo Mimetics

Development of small molecule and peptide mimetics that mimic SMAC can be another novel approach as it is a pro-apoptotic mitochondrial protein that is also an endogenous inhibitor of the IAP family of cellular proteins.

IAPs represent the last line of defense for the cancer cells against apoptosis. Clinically, IAPs have been proven to be a significant factor in cancer cell survival, development, and poor prognosis. Their association with tumor resistance to therapies is considered as one of the important therapeutic targets to selectively induce apoptosis in tumor cells.

The main objective behind designing SMAC mimetics (also called IAP antagonists) is to downregulate cellular IAPs that ultimately induce cancer cell death.

One of the SMAC mimetics, *TL32711,* in its clinical studies on patient-derived tumor xenograft models has successfully led to tumor regression.

Moreover, it also helps in preserving cancer cell sensitivity toward stimulation generated by pro-apoptotic elements like TNF-α or TRAIL, as shown in an in vitro human-derived cancer cell lines.

Collectively, these mimetics has been a great deal in cancer research and therapeutics with great potential to overcome the limitations of current anticancer therapies.

5.7.3 MDM2-p53 Complex

Novel agents that bind MDM2 (mouse double minute 2 homolog) have been developed that displace p53 from the complex, thereby activating the p53 pathway. p53 activation subsequently leads to cell cycle arrest and apoptosis (Vassilev et al. 2004).

Development of such agents as drugs would have the advantage of specifically targeting tumors overexpressing MDM2, leaving the normal cells aside.

5.7.4 Death Receptor Ligands (TNF and TRAIL)

Tumor necrosis factor (TNF) is a well-known anticancer agent in animal models, and has been found to cause death of tumor cells. Interestingly, it acts on the surrounding cells that are associated with feeding the tumor (Stoelcker et al. 2000).

Another important molecule, TRAIL (LeBlanc and Ashkenazi 2003), acts directly on cancer cells and causes apoptosis. It has been well established that TRAIL knockout mice works hand in hand with natural killer (NK) cells in order to keep metastasis from happening (Takeda et al. 2001; Cretney et al. 2002). Moreover, induction of cell death has also been observed in tumor cells from various cancer patients due to the activation of TRAIL-producing macrophages which further release biological molecules that consequently result in upregulation of particular markers on tumor cells (Herbeuval et al. 2003).

5.7.5 Monoclonal Abs (Rituximab) and Apoptosis in Cancer

One of the most potent agents for treatment of lymphocytic cancer such as non-Hodgkin's lymphoma is a monoclonal antibody (Rituximab) against the antigen B220 present in B-cells. This monoclonal antibody mainly acts by inducing apoptosis that ultimately leads to cell death (Cartron et al. 2004).

5.8 Conclusion

Cancer Is a Failure of Apoptosis

Apoptosis or programmed cell death is one of the ways through which the cell cycle is maintained and kept under check. This basic defense mechanism has always been the main reason why cancer cells do not thrive and lead to abnormal cell growth or cancer. For example, cells from the epidermal layer of the skin when exposed to harmful ultraviolet radiations in this case as a part of defense mechanism, programmed cell death is normally activated.

This helps in eliminating those injured cells that otherwise would survive and develop into cancerous growths.

In metastasis or malignant cancer, in order be alive, cells always travel from one organ to the other through the blood system.

This is usually prevented by apoptosis as cells typically "self-destroy" when they lose contact with neighboring cells or the extracellular matrix (Hanahan and Weinberg 2000).

One of the hallmarks of cancer is to escape apoptosis (Hanahan and Weinberg 2011). Cancer cells display many characteristics; for example, they interfere into

cell cycle checkpoints and withstand exposure to cytotoxic agents (Letai 2008) that subsequently lead to their survival. Since apoptosis is an important impediment toward developing cancer, avoiding apoptosis is integral to tumor development and resistance to therapy (Cory et al. 2003; Plati et al. 2011). Cancer cells are capable of escaping apoptosis and continuously dividing. One of the important reasons for this behavior is loss of tumor-suppressor p53.

p53 knockout cells are incapable of sensing DNA damage that drives apoptosis (Hanahan and Weinberg 2000). Anti-apoptotic Bcl-2 family members and IAPs also promote cell proliferation when upregulated (Vo and Letai 2010). Although there has been a huge advancement in understanding the apoptotic pathway and targeting it for therapeutic intervention, the challenges in this area are still manifold. Complex machineries of cancer cells enable them to develop resistance to apoptosis by acquiring new mutations which nullify the drug-induced targeted therapies. For example, apoptosis is initiated in cancer cells when drug inhibits the activity of Bcl-2 family proteins. However, a new mutation generated in the cancer cells may upregulate the caspase inhibitors, and the drug will not be effective anymore (Wong 2011). Therefore, a comprehensive understanding of the apoptotic pathway along with the cross talks among different pro- and anti-apoptotic proteins might open new avenues in devising new strategies to effectively combat cancer.

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Integration of Evolutionary Theory into Cancer Biology and Caspase Signaling

6

Robert D. Grinshpon and A. Clay Clark

Abstract

The caspase family of cysteine proteases consists of evolutionarily ancient regulators of the programmed cell death (PCD) cascade known as apoptosis. Indeed, homologous caspase genes even predate multicellularity. Caspases are persistent throughout all kingdoms of life because they play a crucial role in cellular organization and homeostasis. Cancer occurs when mechanisms that maintain cellular homeostasis are compromised. For example, healthy anchorage-dependent cells commit anoikis – apoptosis triggered by cell detachment – while malignant cancers survive through metastasis by evading PCD signaling. The goal of many cancer therapies is to indirectly initiate PCD, but the future of targeted personalized medicine requires a comprehensive understanding of the genes involved in cancer signaling. Such an approach offers to improve current nonspecific chemotherapies. In this regard, evolutionary theory offers promising new insights into cancer biology because >90% of disease-related genes emerged before the bilaterian radiation, also known as the Cambrian explosion. Thus, the lineage of every extant animal has survived through an evolutionary arms race between growth signaling and tumor suppression while starting with a very similar genetic tool kit. The results are many successful strategies that prevent the occurrence of cancer, and the strategies can be examined through a scientific lens. This review describes how the integration of evolutionary theory serves to enhance our understanding of caspase family signaling in the context of cancer biology.

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Keywords
Caspase · Evolution · Cancer · Apoptosis · Evolutionary medicine · Peto's paradox

6.1 Introduction

The evolution of multicellular life required mechanisms to organize organ tissue, hence the term "organism." Otherwise, aggressive cells with higher proliferative tendencies would outcompete their surrounding cells for life-sustaining resources (Jacqueline et al. 2017). Such behavior is described by the Darwinian model of cancer progression (Greaves and Maley 2012) and the clonal evolution of cancer (Nowell 1976) where "survival of the fittest" selection for opportunistic somatic cells is the driving force toward malignancy (Aktipis et al. 2015).

The beginning of this story is marked by the emergence of the caspase cenancestor, the last common ancestor of all clan CD C14 peptidases, sometime between the development of cellular life and multicellular life around 4.29 and 2.10 billion years ago, respectively (Fig. 6.1). As shown in Fig. 6.1, our best estimates for divergence times and the evolutionary history for humans are reported in million years ago (MYA). Ninety percent of human disease-related genes exist in all descendants of Bilateria, and caspase homologs exist in all descendants of Eukaryota (McLuskey and Mottram 2015). The red circles in Fig. 6.1 represent the relative magnitude of historic meteoric impacts, the yellow bar represents the atmospheric oxygen levels, and the spike at 542 MYA coincides with the end of the Proterozoic eon and the explosion of the earliest multicellular life forms known as the bilaterian radiation (Briggs 2015; Domazet-Lošo and Tautz 2008). Not surprisingly, three caspase genes were found in the oldest known metazoan ancestor, *Porifera* (sponge), as was evidence for DEVDase activity being associated with apoptosis phenotypes (Wiens et al. 2003).

Caspase proteins played a crucial role in fulfilling the organizational requirements for metazoan life, and they have since evolved in humans to function as key regulators of (1) the apoptosis cascade (Redza-Dutordoir and Averill-Bates 2016), (2) the inflammatory immune response (Galluzzi et al. 2016), (3) cellular differentiation (Bell and Megeney 2017; Burgon and Megeney 2017), and (4) various sub-apoptotic functions (Nakajima and Kuranaga 2017) (Fig. 6.2, left). Dysregulation of these functional roles of the caspase family contributes to the development and progression of cancer (Fig. 6.2, right). "Resisting cell death," or evading apoptosis, is one of the six original hallmarks of cancer. Inflammation, which also utilizes caspase activity, was later added to the updated list of ten cancer hallmarks (Mantovani et al. 2008; Hanahan and Weinberg 2011). Solid tumors are known to be heterogeneous populations of differentiated cells (Clevers 2011; Gerlinger et al. 2012; Marusyk and Polyak 2010), and sub-apoptotic caspase activity has been implicated in compensatory apoptosis-induced proliferation (Fogarty and Bergmann 2017). Taken together, caspase signaling is intimately involved in the life-death decisions made by cells.

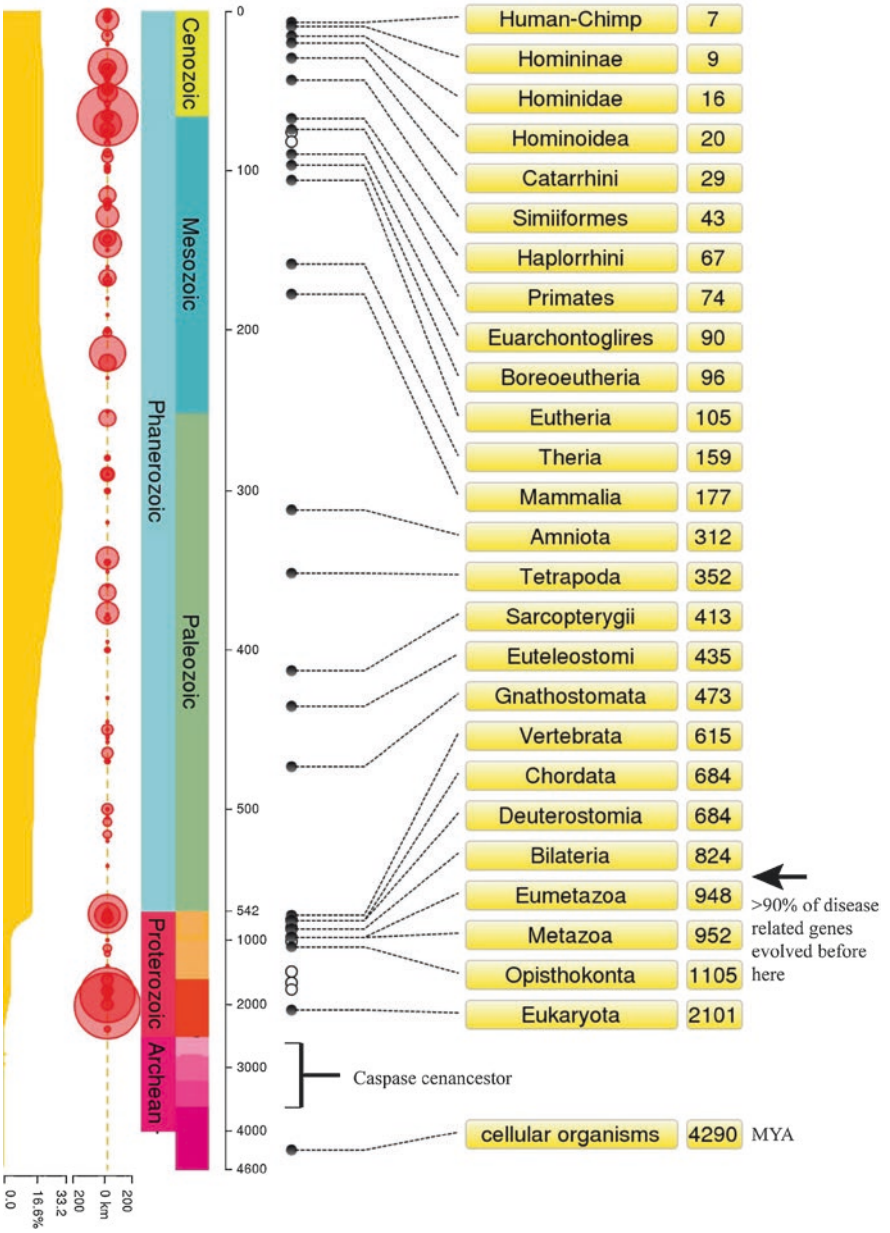

Fig. 6.1 Timeline of human evolution. The evolutionary history of *Homo sapiens* dated in million years ago (MYA). The yellow bar on the left represents atmospheric oxygen, and the spike coincides with bilaterian radiation. The red circles represent the magnitude of historic meteor strikes. The caspase cenancestor evolved sometime between the emergence of life and the eukaryotic cell. Over 90% of disease-related genes evolved before Bilateria

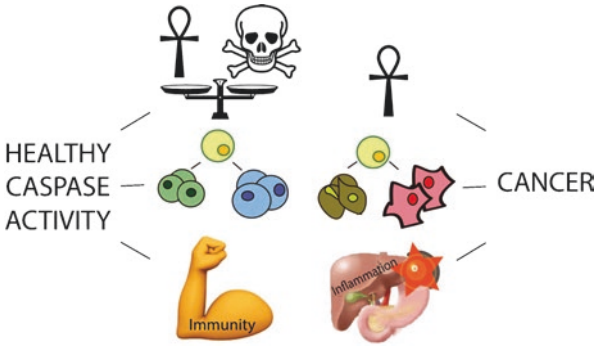

Fig. 6.2 The role of caspases in healthy versus cancer cells. The images on the left depict caspase functions in healthy cells, such as cellular homeostasis, cell differentiation, and proper immune response. Dysregulation of normal caspase activity leads to hallmarks of cancer, such as evasion of apoptosis, abnormal cell morphology, and inflammation (right panel)

6.1.1 Caspases and Apoptosis

Apoptosis is one of many programmed cell death (PCD) phenotypes, and it is an active process of self-destruction that initiates in the absence of life-sustaining signals. PCD cascades are genetically encoded and latently poised to dismantle the cell when viability is compromised. Thus, life itself is an active process of preventing death. The apoptosis phenotype is characterized by chromatin condensation, chromosomal DNA fragmentation, membrane blebbing, cell shrinkage, phosphatidylserine display, and disassembly of the cell into membrane-enclosed vesicles (Kerr et al. 1972). The intricacies of caspase activation and signaling cascades have been reviewed extensively elsewhere (Budihardjo et al. 1999; Parrish et al. 2013; Redza-Dutordoir and Averill-Bates 2016). We focus here on how caspase signaling has changed over time and why understanding the evolutionary history of caspase signaling is relevant to cancer biology.

The term "caspase," or (c)ysteinyl (asp)artate-specific prote(ase), is derived from its enzymatic function of facilitating the hydrolysis of target peptide substrates with a catalytic cysteine-histidine dyad and C-terminal to aspartate residues. Caspases also participate in many diverse non-apoptotic cellular functions (Connolly et al. 2014), and recent studies of the caspase degradome document 777 putative caspase substrates in the CASBAH database of human caspase substrates (Lüthi and Martin 2007). The repercussions of substrate cleavage, however, are unknown for many of the entries (Julien and Wells 2017).

Protease families are organized in the MEROPS database based on statistically significant sequence similarity (Rawlings et al. 2016). Caspases are categorized into the C14A subfamily of the clan CD cysteine proteases, while the more recently discovered meta-, ortho-, and para-caspases comprise the C14B subfamily (Cade and Clark 2015; Klemenčič and Funk 2017). Clan C14B enzymes are distant homologs that participate in cell fate regulation, but they are technically not "caspases" since they prefer to cleave after Arg and Lys residues (Vercammen et al. 2004; Enoksson and Salvesen 2010). Clan C14 is the only family of clan CD proteases

that is represented in organisms across the entire tree of life (McLuskey and Mottram 2015), and much of the machinery for the intrinsic and extrinsic apoptosis pathways has been conserved from *Acropora millepora* (pre-bilaterian coral) to human (Moya et al. 2016). Crystallographic models of ~4-billion-year-old ancestral protein state resurrections of thioredoxins show that protein structure is generally conserved despite evolutionary distances (Ingles-Prieto et al. 2013). The data suggest that the caspase-hemoglobinase structural scaffold evolved for functional purposes and that the overall fold has been conserved throughout the evolution of the modern caspase subfamilies. Each member of the human caspase family has retained the canonical caspase structure-function while evolving discrete regulatory mechanisms and disparate cellular roles, albeit with some redundancy (Berger et al. 2006).

On the apoptotic side of the family, the effector caspases (3, 6, 7) evolved from the initiators (Fig. 6.3). All C14A caspases are obligate dimers. The initiator caspases are synthesized as stable monomers in solution, and they form an active caspase dimer upon receiving apoptotic stimuli. The induced proximity model proposes that dimerization is facilitated by the increased concentration of initiator caspases due to spatial localization by their DED and CARD recruitment domains (Salvesen and Dixit 1999; Boatright et al. 2003). Dimerization alone is sufficient for basal-level activity in the initiator caspases; however, the effector caspases require cleavage of the intersubunit linker (IL), which results in rearrangement of the active site loops to form a catalytically competent enzyme (Clark 2016). The effector caspases do not require recruitment domains because they exist as stable, yet inactive, dimers in solution. At present, it is not clear how the common ancestor of effector caspases evolved a stable dimer interface as well as regulatory mechanisms that prevented apoptotic induction at the same time. The simultaneous evolution of the effector procaspase dimer and inactivation of the dimer is an intriguing problem.

As mentioned above, caspases transmit cell signals by cleaving target substrates. An analysis of caspase target substrates in humans, mice, fruit flies, and nematodes revealed a hierarchical order of evolutionary conservation (Crawford et al. 2012). That is, the signaling pathway that is disrupted is more conserved than the substrate target within that pathway, and the cut location and substrate motif are even less conserved. Caspases are well known for their role in the apoptosis pathway; thus, it is not surprising that ectopic expression of human, insect, and nematode caspases induces apoptotic phenotypes in budding yeast (Puryer and Hawkins 2006; Lisa-Santamaría et al. 2009), and planarian BCL-2 homologs can regulate mitochondrial outer membrane permeabilization (MOMP) in mammalian cells (Bender et al. 2012). Given the evidence, it is likely that caspase proteins have been involved in regulating cell death pathways at least since the advent of multicellular life.

In humans, caspase-3 has evolved to be the main executioner of apoptosis, and many cancer therapies ultimately kill cancer cells by initiating the apoptotic cascade (Flanagan et al. 2016; Fitzwalter and Thorburn 2017). For example, chemotherapy induces irreparable DNA damage, which in turn initiates apoptosis indirectly via accumulation of p53 (Kaufmann and Earnshaw 2000; MacKenzie et al. 2010). However, chemotherapy is not always successful because cancer stem cells can selectively evolve chemoresistance (Abdullah and Chow 2013).

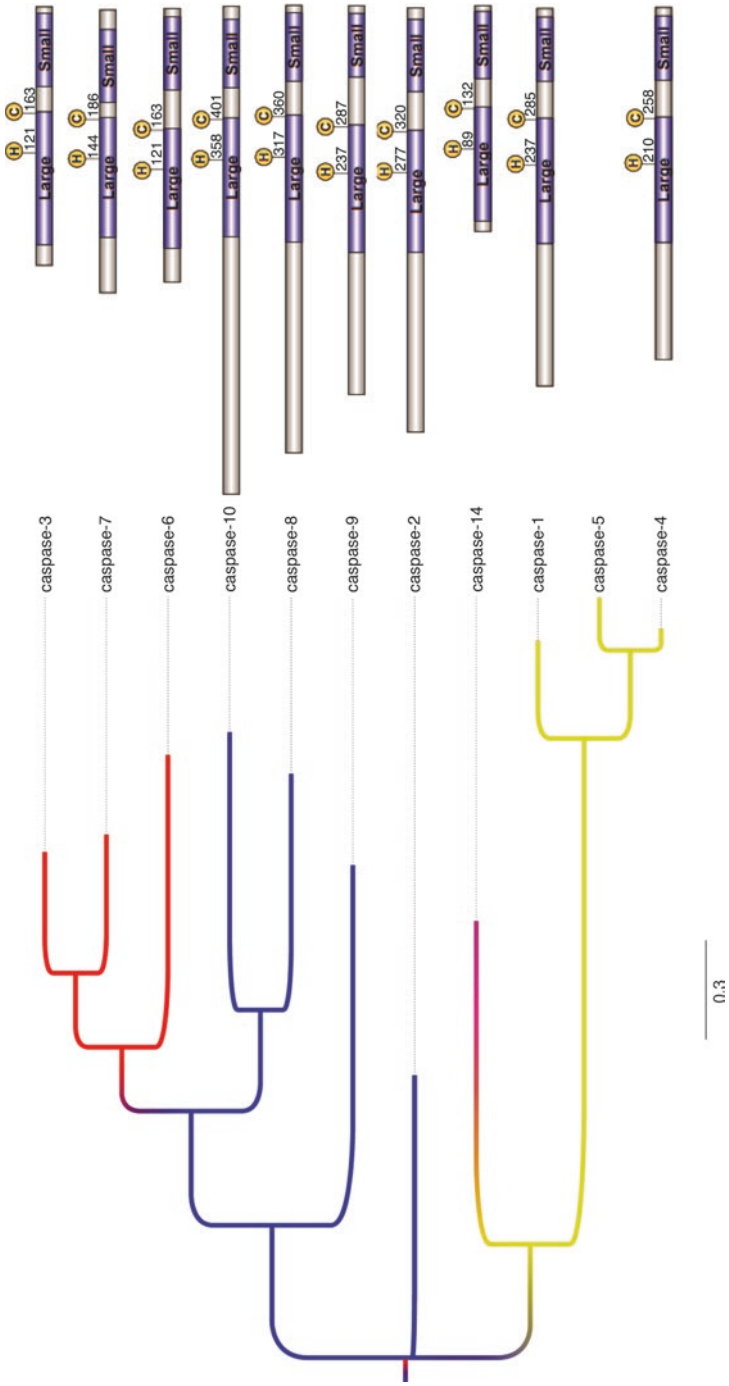

Fig. 6.3 Human caspase family tree. The human caspase genes have evolved discrete functions in the cell while conserving the structural-functional feature of the caspases cenancestor that defines a "caspase." The above caspases are broadly categorized by their most well-known role. The phylogenetic tree is an estimation of the relationships among the human caspase genes, and the branch lengths are an estimation of divergence time

Chemoresistant cells are essentially "apoptosis resistant." However, the number of mechanisms by which a cell can evade apoptosis is yet to be determined. For example, $CD133^+$ cancer stem cells are a marker for tumors with strong chemoresistance (Liu et al. 2006). Not surprisingly, the anti-apoptotic genes (FLIP, BCL-2, and BCL-XL) and the inhibitors of apoptosis (IAP) family members (XIAP, cIAP1, cIAP2, NAIP, and survivin) are expressed at significantly higher levels in $CD133^+$ cells (Liu et al. 2006). In addition, upregulation of the eukaryotic translation initiation factor 5A2 (EIF5A2) is another mechanism of chemoresistance in esophageal squamous cell carcinomas. The increased levels of EIF5A2 limits caspase 8 activation by altering protein expression (Yang et al. 2015). Finally, genetic variations in apoptosis-related genes are also associated with increased susceptibility to certain cancers (Ghavami et al. 2009; Xie et al. 2016). Molecular evolution and caspase polymorphisms in cancer prognosis are discussed in the following sections.

In summary, multicellular life required mechanisms such as apoptosis to enhance cellular cooperation. The core apoptotic machinery is generally conserved across multicellular organisms, and dysregulation of PCD signaling is a hallmark of cancer. The caspase structure-function is highly conserved, but each caspase has evolved discrete regulatory mechanisms to fine-tune activity as required for non-apoptotic versus apoptotic events. Understanding how evolutionarily distant model organisms regulate oncogenic growth, specifically how caspases regulate apoptosis, is a promising avenue for cancer biology research. In the next section, we will discuss how evolutionary theory can be applied to understand the cross section of caspase function and cancer biology.

6.2 Evolutionary Biology: Why Do We Care About Caspases in Other Species?

6.2.1 Phylogenetic Inference of Evolutionary Relationships

The term "phylogenetics" dates back to Carl Linnaeus' original classification system where "phylum" was the broadest level of categorization (Brown 2002). The goal of phylogenetics is to estimate ancestor-descendant relationships using a coalescent process (Kingman 1982), which involves using a sample of existing objects to make inferences about their past. The concept of inferring such relationships has been adapted for broad uses in scientific research. For example, phylogenomics was developed to predict the function of a gene from its primary sequence (Eisen 1998), and phylogeography attempts to reconcile microevolutionary changes with macroevolutionary phenotypes over geographical distances and time (Avise et al. 1987). Other phylo-sciences and their relevance to cancer biology are discussed further in this section.

The mechanism of clonal expansion in cancer evolution was first described in 1976 where neoplasms originate from a single cell, and then cellular heterogeneity accrues along with tumor progression (Nowell 1976). Although scientists were aware of histological heterogeneity at the time, little emphasis had been placed on the evolutionary

process behind the formation of tumor cell populations (Clevers 2011). The heterogeneity and clonal nature of cancer fit the framework for phylogenetic analysis, which has been applied to predict the clonal frequency of tumor cell populations (Malikic et al. 2015). The record of a tumor's evolutionary trajectory is maintained within the genetic landscape, so tumor phylogenetics can be used to identify the root cause of metastasis (Naxerova and Jain 2015). Phylomedicine is another example application being explored in light of the recent interests in having one's genome sequenced. In this case, small sequence variations among the general population are analyzed for their disposition to genetic diseases such as cancer (Kumar et al. 2011).

6.2.2 Molecular Evolution and Functional Genomics

Researchers began comparing protein sequences to homologs in other species as soon as the ability to determine amino acid sequence became available (Wilson et al. 1977). The observed linear trend of sequence evolution became the basis for the molecular clock hypothesis (MCH) (Thorpe 1982). The MCH is a model that describes the relationship between the degree of consensus of two orthologous genes as a function of time since they diverged. However, it was also observed that the rate of evolution is not the same in all genes. The primary parameter that determines the evolutionary rate of a gene is its expression levels, not the importance of the function (Zhang and Yang 2015). The rate of evolution is also inversely related to the age of the gene (Albà and Castresana 2004), and the rate can vary at each amino acid position along the protein (Echave et al. 2016). Understanding the site-specific nature of evolution, and how proteins evolve naturally according to neutral mutation theory (Kimura 1968), will be important to better understand the physical manifestation of somatic mutations that drive cancer.

Protein sequence space and evolution have been described as a walk from one functional protein to another in the space of all possible protein sequences (Smith 1970). Evolution occurs at the genetic level, but the expressed gene product interfaces with the physical environment. Because proteins exist as an ensemble of conformational states (Boehr et al. 2009), non-silent mutations to the genome alter the energy landscape of the expressed protein. Mutational perturbations that affect protein dynamics, by shifting the equilibrium of the conformational ensemble, are relevant to the clinically observed cancer mutations that confer drug resistance (Wilson et al. 2015; Freed et al. 2016).

The order of mutations can drastically change the evolutionary trajectory of a protein and is referred to as within-protein epistasis. More specifically, epistasis is defined as the nonadditive effect on protein fitness from two or more mutations (Fig. 6.4) (Starr and Thornton 2016). The consequence of intra-protein epistasis is that the effect of any mutation will depend on the energetic background in which it appears, such that the new mutation alters how subsequent mutations contribute to the total free energy (Weinreich et al. 2005). An energetically unfavorable mutation, for example, may ultimately be acceptable due to subsequent stabilizing mutations at distal locations, an effect that has been described similarly to a "stokes shift" (Pollock et al. 2012).

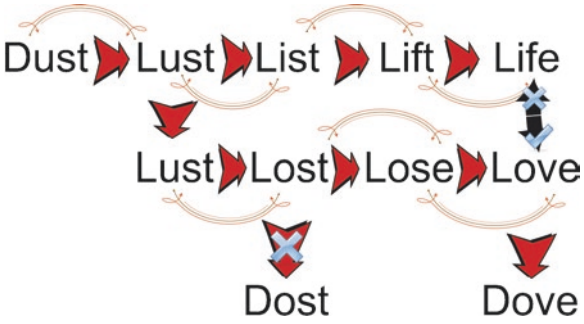

Fig. 6.4 Conceptual model of within-protein epistasis. In this model, words that have a meaning are considered functional. New mutations must form new words with meaning to maintain function. Each sequential mutation can be permissive or restrictive to letters that can be accepted at the other 3 positions. The common ancestor of LIFE and LOVE is DUST. The word LUST represents a gene duplication event. The mutation from LUST to LOST is a restrictive mutation for D at the first position, whereas the following mutation from LOST to LOSE is permissive for D at the first position

The concept of within-protein epistasis is illustrated in Fig. 6.4. In this example, each mutation must generate a word in the English dictionary to represent the preservation of protein function: each black arrow represents a mutation, and each word is a functional state of a hypothetical protein. Mutations with an X result in nonsense or "lack function." In this example, DUST is the common ancestor of LIFE and LOVE. Although there are many possible words that can be made from evolving DUST, the majority of mutations will be deleterious. Each mutation can be considered as permissive or restrictive, relative to the other amino acid positions (Harms and Thornton 2010). In Fig. 6.4, for example, the L in LOST cannot mutate back to D because the U ➔ O mutation was restrictive. However, the following T ➔ E mutation was again permissive for D at the first position to form DOVE. The concept of epistasis is important to keep in mind when researching the structure-function relationship of a protein. Traditional biochemistry experiments that employ site-directed mutagenesis often assess the effects of horizontal mutations, that is, swapping specific sites in relative homologs. In many cases, however, horizontal mutations do not account for the effects of epistatic interactions. An example of an effective horizontal mutation as well one that is not effective is shown in Fig. 6.4: LIFE can mutate to LIVE, but LOVE cannot mutate to LOFE.

In contrast to horizontal mutations, experiments that employ vertical mutations computationally derive amino acid changes through a phylogenetic technique called ancestral state reconstruction (ASR). An ASR adds evolutionary time to the structure-function relationship. Since evolution tends to preserve the functionality of extant proteins, one can presume that the nodes of a gene tree represent a functional ancestral state (Pauling and Zuckerkandl 1963).

Therapeutic cancer drugs are typically designed to target the biological function of enzymes that have been deregulated by driver mutations, such as the constitutively active Bcr/Abl kinase and Gleevec®. Likewise, "resistance mutations" recover

the function of the drug target, despite the presence of the drug by interfering with the drug's binding site. In a less likely scenario, a mutation increases the biological function of the drug target to overcome the diminishing effect of the drug (Friedman 2016). An ASR of the human oncogenes Src and Abl was executed to examine the mechanism of the anticancer drug Gleevec® (Wilson et al. 2015), which is a potent inhibitor of Abl kinase but not the closely related Src kinase (Lin et al. 2013). The ASR experiment yielded an array of ancestral states along the evolutionary trajectory of Abl that exhibited intermediate affinities for Gleevec®, so the evolutionary changes in Abl from one state to the next could be associated with the changes in the efficacy of the drug. The results showed that Abl altered the induced-fit equilibrium toward binding the drug. In addition, the data showed that the clinically observed Abl mutation, T315I, disrupts the free energy landscape for docking the drug (Wilson et al. 2015). Altogether, the ASR studies of Abl showed that understanding the natural evolutionary constraints of a protein serves as a starting point for assessing the functional implications of cancer-driving mutations.

In evolutionary functional genomics (EFG) studies, researchers examine the function of genes using molecular evolution theory and sequence data (Gu 2003). A great deal of effort has focused on understanding evolutionary relationships through sequence comparison (Sikosek and Chan 2014) because the mutation rates of individual amino acids change when new functions evolve (Fitch and Markowitz 1970). The effective rate of protein sequence evolution is determined by comparing the number of non-synonymous nucleotide substitutions (K_a) per non-synonymous site, where K_a mutations are amino acid changing while synonymous mutations (K_s) are silent. The protein signaling cascade in the cell death program (apoptosis) is an excellent example to illustrate the power of EFG. Typically, 34 mammalian genes, split into four subcategories, are involved in the intrinsic apoptosis cascade (Fig. 6.5). The ratio of non-synonymous (amino acid changing) to synonymous (silent) substitutions (K_a/K_s) among the 34 genes was determined for primates, rodents, and carnivores (Vallender and Lahn 2006). From such data, the ratio of K_a/K_s can be used to estimate site-specific rate variation (SSRV) or the rate at which a position has accepted changes in amino acids. The SSRV is used to locate evolutionary hot spots as well as to detect evolutionary functional divergence. A shift in SSRV is indicative of a change in evolutionary constraints (Gaucher et al. 2002). For example, functional divergence analysis of SSRV in the caspase family revealed that the evolution of major caspase-mediated pathways has been facilitated by gene duplications (Wang and Gu 2001; Zhang 2003). Indeed, the long functional branch lengths of caspases 2, 3, and 6 are evidence of extensive altered functional constraints as a result of evolving specialized roles in human apoptosis (Wang and Gu 2001). The K_a/K_s ratio is significantly higher in human caspase-dependent intrinsic apoptosis genes relative to rodents and carnivores and even relative to closely related primates. Caspase 3, in particular, exhibits strong evolutionary rate disparities when compared to other primates (Vallender and Lahn 2006). Evidence has shown that cells of the close relative *Pan troglodytes* (chimpanzee) exhibit relatively increased sensitivity to apoptosis, which could explain why humans have increased susceptibility to cancer, larger brain sizes, and longer life spans (Arora et al. 2012). Such

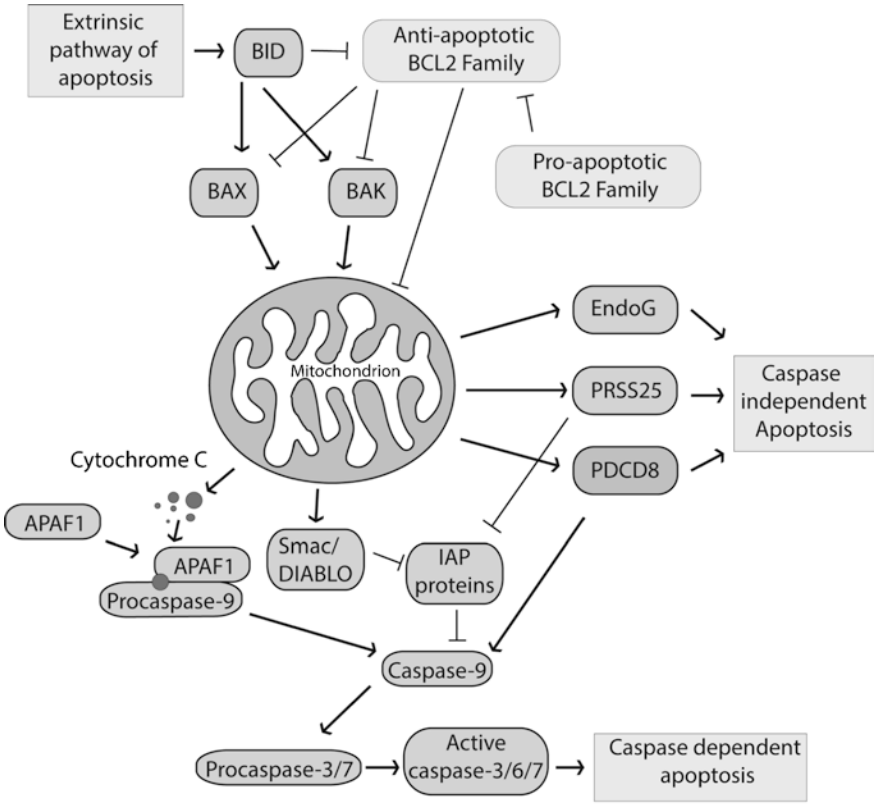

Fig. 6.5 The intrinsic pathway of apoptosis. The four families of proteins that have coevolved to regulate the mechanism of cell death: the IAP family, the caspase family, the pro-apoptotic BCL-2 family, and the anti-apoptotic BCL-2 family

comparative analyses provide insight into how small differences in closely related genomes manifest phenotypically.

6.2.3 Evolutionary Medicine

"Evolutionary medicine" is a relatively new and underexplored umbrella term for research that incorporates evolutionary theory into modern medicinal research (Aktipis et al. 2013). Four principles highlight the use for evolutionary medicine in furthering our understanding of cancer: (1) neoplasms are heterogeneous populations of cells that accumulate mutations via somatic evolution; (2) cell fitness depends on genotype, phenotype, and surrounding ecological factors; (3) innate cancer defense mechanisms evolved over a billion years ago; and (4) evolutionary medicine explains the trade-offs between the risks associated with cancer and cell maintenance (Aktipis et al. 2013). Evolutionary medicine is being applied to design

more efficient interventions for cancer management and prevention because the manifestation of cancer can be described by an evolutionary process on two different levels, emergence at the population level and progression within the individual host (Hochberg et al. 2013). Such insight has led to novel avenues of research for early detection and treatment of cancers (Lemaître et al. 2015; Ujvari et al. 2016).

As previously established, the fundamental life-sustaining mechanisms of a cell are regulated by evolutionary ancient networks of signaling proteins (Bussey et al. 2017). Phylostratigraphy is a discipline of evolutionary biology that utilizes phylogenetic methods to determine the age of such disease-related genes. The information is relevant to understanding tumorigenesis because information on the age of a gene also provides information on the evolution of gene networks (Cheng et al. 2014). Cancer-related genes tend to have a longer evolutionary history with an inverse rate of evolutionary change (Albà and Castresana 2004). Approximately 60% of 22,845 human genes can be traced back to the early origins of life and the emergence of eukaryotic cells (Domazet-Lošo et al. 2007). The evolution of multicellularity (Eumetazoa) is also associated with a spike in the emergence of genes. Only 13% of human genes are unique to the mammalian lineage, and only 0.6% of those genes are disease related (Domazet-Lošo and Tautz 2008). Indeed, over 90% of disease-related genes emerged before the bilaterian radiation (Fig. 6.1) (Domazet-Lošo and Tautz 2008). A list of genes that are implicated in human cancer, with homologs present in the sponge genome, provides a starting point for discovering new drug targets relevant to cancer (Ćetković et al. 2018).

Interestingly, the emergence of "caretaker" genes spiked around the same time as the appearance of Eukaryota, and the emergence of "gatekeeper" genes spiked around the same time as the appearance of Eumetazoa (Domazet-Lošo and Tautz 2010). The trend in gene appearances is consistent with the tumor progression model. The model states that mutations in critical caretaker genes increase the probability of genetic instability and that such mutations tend to occur before gatekeeper function is compromised (Nowell 1986).

The atavistic theory of cancer posits that cancer is a process akin to rewinding evolution where the loss of critically evolved regulatory features causes the behavior of the cell to revert to the unchecked growth tendencies observed in unicellular organisms (Davies and Lineweaver 2011; Chen et al. 2015). The atavistic theory of cancer provides an explanation for the paradoxical observation that neoplasms can quickly gain new behaviors that promote carcinogenesis shortly after failing to maintain normal housekeeping and regulatory functions. Supporting evidence showed that the hallmarks of cell transformation in solid tumors correlate with a preferential shift of gene expression toward genes that originated in prokaryotes (Trigos et al. 2017).

Evolutionary theory also suggests that resources allocated to reproduction by an organism is constrained by other competing needs, such as cell maintenance and growth, and that the ability to keep cancerous lesions at bay diminishes once an individual has reached an age of low Darwinian fitness (Heino and Kaitala 1999). Cancer is a disease associated with age, which explains why the majority of cancer patients are past the age of their reproductive prime (Balducci and Ershler 2005).

Although the occurrence of cancer is *relatively* rare, most adult humans maintain potentially malignant tumors that are difficult to detect (Aktipis and Nesse 2013). However, many people live for decades without developing malignant cancer (Bissell and Hines 2011). The latter observations have led to the hypothesis that natural selection promotes mechanisms that hold tumors in check through the age of an organisms' reproductive prime (Kirkwood 2005; DeGregori 2011).

Cancer progression is associated with increasing genotypic and phenotypic heterogeneities (Gerlinger et al. 2012). Heterogeneity within a tumor can interfere with personalized cancer therapies that were designed based on a single biopsy because expression patterns with both good and poor prognoses have been detected within different regions of the same tumor (Gerlinger et al. 2012). Difficulty in pinpointing the source that is driving the cancer, and the likelihood of developing chemoresistant cells, also increases over time. Altogether, studies in evolutionary medicine suggest that cancer prevention measures should be implemented before reaching an age of post-Darwinian fitness because early detection and treatment can curb cancer progression and prevent chemoresistance.

6.2.4 Peto's Paradox and Comparative Genomics

Comparative oncology is another subbranch of evolutionary medicine that has been steadily gaining traction. Researchers attempt to explain how evolutionary differences among species affect the diversity, incidence, and lethality of various cancers by analyzing the dynamics of carcinogenesis in different tissues and across multiple species. Interest in comparative oncology arose from observations such as "Peto's paradox," or the inconsistent relationship between the predicted and the observed rates of cancer in species with contrasting body sizes (Nunney et al. 2015). For example, since it has been observed that the probability of developing cancer increases with age and number of cell divisions, then the predicted rate of cancer would be higher in larger and longer-lived animals like elephants. However, this is not the case (Abegglen et al. 2015).

A comparative study of several mammalian species sought to identify the mechanisms of cancer resistance in elephants by investigating alterations in cancer-related genes. Elephants maintain a lower than expected risk of cancer compared to humans despite their larger body size and long life span (Greaves and Ermini 2015). The analysis suggested that the >20 copies (40 alleles) of the TP53 gene in elephants, compared to the single copy in humans, is responsible for their decreased susceptibility to cancer (Abegglen et al. 2015). Another project assessed dermal fibroblasts from African and Asian elephants, rock hyrax, aardvark, and armadillo. After treatment with DNA damage-inducing agents, measures of TP53 activation, caspase 3/7 activity, and levels of apoptosis suggested that elephants more readily upregulate TP53, and induce apoptosis at lower levels of DNA damage, compared to the other test animals (Sulak et al. 2016). The fact that Peto's paradox can be understood through adaptive evolution allows for the application of evolutionary and population genetics theories to investigate differences in the cancer biology among taxa.

Peto's paradox also applies to the comparison of organs within the body because organs with more stem cells and more cell turnover generally develop cancer more frequently (Tomasetti and Vogelstein 2015; Nunney 2013). Characteristics of cancers, such as the number of driver mutations required to form a tumor, range greatly between the tissue and the species of its origin (Vogelstein et al. 2013). An understanding of the tissue-specific differences through comparative oncology will help in the design of improved strategies for cancer prevention and treatment.

Comparative oncology is also being applied to learn about osteosarcomas, a cancer that commonly occurs in adult canines and adolescent humans, with similar etiological history based on genome-wide expression profiles. Interestingly, larger canine breeds are more susceptible to osteosarcomas, but the genetic determinants are unknown (Varshney et al. 2016). Understanding why smaller breeds are less susceptible may provide insight into which signaling pathways are involved in regulating childhood osteosarcomas. The application of comparative genomics extends beyond bioinformatics. For example, canine and human metabolisms process certain drugs similarly, such as the anticancer drug iniparib (Saba et al. 2016), a drug candidate for cancer treatment that was removed from the market after passing clinical trials (Mateo et al. 2013). A follow-up study was done on household pets that developed spontaneous cancers where the patients were assessed for pharmacokinetic exposure and tolerability to iniparib. Relevant concentrations of iniparib, or its metabolites, were not detected in tumor tissues at any dose. The results quash the possibility of a clinical response in canines and offer an explanation as to why iniparib was ineffective in humans (Saba et al. 2016). The study highlights the potential of comparative oncology because such dose tolerability experiments in humans are unethical.

6.3 Caspase Signaling and Cancer

6.3.1 Origins of the Apoptotic Pathway

The origin of caspase proteins is debatable. New genes are not "invented" by evolution, but homologous components necessary to orchestrate programmed cell death (PCD) in prokaryotes were not discovered until recently (Huettenbrenner et al. 2003). It was shown that up to 90% of cells undergo a form of cell death during fruiting body formation of *Myxobacteria*, and the unicellular parasite *Dictyostelium discoideum* displays cell death phenotypes reminiscent of apoptosis (Huettenbrenner et al. 2003). The evidence for supporting apoptotic origins in bacteria has been mounting since the discovery of such apoptosis-like phenotypes (Bayles 2014).

Endosymbiosis occurs often in nature; however, the variety of extant flora and fauna is a testament to the successful marriage of eukaryotes and α-proteobacteria. The Cid/Lrg family of proteins was transferred to the eukaryotic host after endosymbiosis and evolved into the Bcl-2 family (Embley and Martin 2006). Bacteria have conserved holin-like proteins (CidA and LrgA) that were first discovered in *Staphylococcus aureus* (Bayles 2007), and the proteins function analogously to the Bcl-2 family proteins by depolarizing membranes upon their insertion. The downstream targets of

bacterial holins, however, are peptidoglycan hydrolases instead of caspases, likely due to the compositional differences in bacterial membranes (Bayles 2014). Interestingly, human Bax and Bak are capable of inducing holin-dependent cell death when expressed in *E. coli*, but Bax mutants with reduced apoptotic potential are no longer capable of inducing PCD (Pang et al. 2011). The hypothesis, that a universal mechanism underlying the control of PCD, is substantiated by the ability of proteins in evolutionarily distant organisms to retain their functional role (Pang et al. 2011).

Further evidence for the unicellular origins of apoptosis was found in *Plasmodium berghei* and *Plasmodium falciparum* ookinetes, which are protozoan parasites used in a rodent malaria model. The organisms exhibited classic apoptosis hallmarks, including DNA fragmentation with positive TUNEL staining, caspase-like activity, and phosphatidylserine translocation. The absence of orange fluorescent JC-1 aggregates in apoptotic cells implied that mitochondrial outer membrane permeabilization (MOMP) had occurred (Arambage et al. 2009). Genes encoding caspases in protozoans had not yet been discovered at the time of these studies, so the target of the caspase-specific inhibitors was proposed to be a metacaspase in *P. falciparum*. Disruption of *P. berghei* metacaspases PbMC1 and PbMC2, however, did not result in decreased apoptosis, so the search for the apoptosis-inducing gene in *P. berghei* continues (Arambage et al. 2009).

Mitochondria are integral to the regulation of cellular homeostasis in humans, and many of the components involved in the mitochondrial pathway of apoptosis (Fig. 6.5, intrinsic pathway) are highly conserved throughout metazoan evolution, such as the anti-apoptotic Bcl-2 family. The Bcl-2 family sequesters the Bcl-2-homology (BH) domain 3 (BH3) of pro-apoptotic Bcl-2-family members, and Bcl-2 is upregulated in many cancers (Akl et al. 2014). However, the intricacies of the apoptosis cascade vary by degree of taxonomic separation. For example, MOMP is not a universal apoptotic phenotype in metazoans, and the degree to which MOMP is conserved is unknown (Lee et al. 2014). Mitochondrial-mediated apoptosis is characterized by MOMP and the activation of caspases by cytochrome C. MOMP has been observed in platyhelminthes, but not in Porifera, Cnidaria, Nematoda, Arthropoda, and Echinodermata (Lee et al. 2014). Interestingly, trypanosomatids are eukaryotic protozoans that lack the canonical machinery to carry out apoptosis (Bcl-2, caspases, and TNF-related receptors); however, they still exhibit many of the common apoptosis phenotypes during PCD (cell shrinkage, membrane blebbing, and chromatin condensation) (Smirlis and Soteriadou 2011). An understanding of how apoptosis is regulated in trypanosomatid may shed light on the evolutionary origin of the metazoan apoptosis signaling network. Such studies may yield alternative strategies to induce apoptosis.

Only one copy of the Apaf-1 gene is consistently present in the vertebrate lineage; however, the presence of multiple Apaf-1 homologs in the common ancestor of bilaterians suggests that several mechanisms to activate intrinsic apoptosis may have existed previously (Zmasek et al. 2007). For example, MOMP can alternatively trigger the TNF-dependent pyroptosis cell death pathway independent of caspase activity (Giampazolias et al. 2017). Exploiting alternative approaches to induce cell death may lead to novel strategies to combat cancer.

6.3.2 Diversity in the Caspase Family

Much of our knowledge about invertebrate apoptosis is based on two prominent model organisms (*D. melanogaster* and *C. elegans*). By happenstance, the role for the mitochondria and MOMP release of cytochrome C during apoptosis was diminished. Thus, it was previously thought that the intrinsic apoptosis signaling pathway was unique to vertebrates (Bridgham et al. 2003). It was later shown, however, that MOMP, cytochrome C activation of caspases, and subsequent apoptosis occur in planaria (platyhelminthes), which is an evolutionarily older species than *C. elegans* or Drosophila (Bender et al. 2012). We now know that the *C. elegans* apoptosis pathway was shaped by two caspase gene deletion events (Weill et al. 2005). The initiation of apoptosis via mitochondrial signaling has since been observed in bivalves (Estevez-Calvar et al. 2013) and, most recently, in coral (Moya et al. 2016). Each metazoan lineage has evolved a unique repertoire of expressed caspase proteins that range in function, regulation, and activity. For example, the number of caspase genes ranges from five in *C. elegans* to fifty-three in amphioxus, although the functions of the caspase genes in amphioxus remain mostly uncharacterized (Zmasek et al. 2007). Our current understanding of gene evolution and apoptosis signaling in multiple species suggests that expanding research efforts in comparative evolutionary biology will revise the conclusions drawn from earlier studies that relied on a small number of model organisms.

6.3.3 Caspases in Cancer

Although caspase 3 is considered the main executioner of apoptosis, additional cell death programs that are regulated by caspases can also be targeted to kill cancer cells. Caspase 1, for example, is known for its roles in the immune response and in inflammation (Galluzzi et al. 2016), as well as an alternative PCD phenotype called pyroptosis (Bergsbaken et al. 2009). Val-boroPro is an inhibitor of the S9 family of serine proteases, and it induces tumor regression in various mouse models of cancer through immune-mediated responses (Okondo et al. 2017). Inhibition of DPP8/9 S9 family proteases leads to the activation of procaspase-1, which was shown to induce pyroptosis in monocytes and macrophages (Okondo et al. 2017). In addition, an anti-tumorigenic p53 transcription factor homolog, p63, was found to be directly correlated to caspase 1 expression and positive cancer survival outcome (Celardo et al. 2013). Finally, cancer immunotherapy has proven to be a viable option for cancer treatment (Morrissey et al. 2016), suggesting that stimulating caspase-1-dependent cell death in cancer cells may also be a viable therapeutic strategy.

Cytochrome P450 1B1 is a universal tumor biomarker, and increased expression leads to increased proliferative, migratory, and invasive potential in prostate cancer cells (Chang et al. 2017). Knocking down cytochrome P450 1B1 with small hairpin RNAs reduced tumorigenic activities in prostate tumor xenograft mouse models, and the expression and apoptotic activity related to caspase 1 was found to be inversely related to cytochrome P450 1B1 expression (Chang et al. 2017). The disruption of

mitochondrial DNA metabolism by inhibiting LigIIIa has differential effects in normal versus malignant cells where a caspase-1-dependent apoptotic pathway is activated only in malignant cells (Sallmyr et al. 2016). Collectively, the studies show that a better understanding of the conditions that lead to caspase-1-dependent apoptosis, versus inflammation, will help to improve cancer immunotherapy treatment strategies.

The initiator caspases (2, 9, 8, and 10) are distinguished by their long prodomains that contain recruitment domains (death domain (DD), death effector domain (DED), or caspase activation and recruitment domain (CARD)). The recruitment domain results in zymogen activation via an "induced proximity model," leading to zymogen dimerization and subsequent cleavage of effector caspases (3, 6, 7) (Boatright et al. 2003). Although caspase 2 maintains the highest evolutionary conservation, its function was relatively unknown until recently (Kumar 2009). Caspase 2 is known to limit oxidative stress and chromosomal instability, and $Casp2^{-/-}$ deficient mice exhibit increased susceptibility to the development of hepatocellular carcinoma with accelerated tumor progression (Shalini et al. 2016). Even caspase 4, a chordate-specific gene duplicate and regulator of ER stress-induced apoptosis, was found to be actively responsible for inducing apoptosis in human colon cancer cells after saikosaponin-a treatment in a mouse xenograft model (Kang et al. 2017).

Caspase 6 is better known for its role in Alzheimer's disease; however, caspase 6 activity has also been implicated in certain cancers, such as gastric and colon cancer (Yoo et al. 2004; Suboj et al. 2012). For example, colorectal cancer cells notably escape Fas-mediated cell death in the presence of Fas ligand (FasL). However, FasL/Fas-dependent cell death was observed when ARK5 antisense RNA was introduced into SW480 cells (Suzuki et al. 2004). ARK5 is a tumor malignancy-related factor downstream of Akt that putatively regulates caspase 6 activity via phosphorylation at Ser^{257} (Suzuki et al. 2004).

Caspases 8, caspase 10, and CFLAR (caspase 8 and FADD-like apoptosis regulator) are in close proximity on human chromosome 2, locus q33–q34, which has been dubbed the caspase 8 subfamily locus (Eckhart et al. 2008). Duplicate genes that remain in close proximity suggest that the duplication event was relatively recent. Knockout models of caspase 8 have shown that RIP kinase (receptor interacting protein kinase) proteins also initiate the necroptosis pathway (Salvesen and Walsh 2014). The pro-apoptotic and pro-survival functions of caspase 8 are regulated by an interaction with the product of the CFLAR gene, the pseudo-caspase cFLIP (cellular FLICE inhibitory protein), although the mechanisms that regulate substrate specificity of caspase 8 are not known. Caspase 8 functions as a key switch between necroptosis and apoptosis, depending on formation of a heterodimer with cFLIP or a homodimer (Boatright et al. 2003). Interactions between the paralogs caspase 8 and cFLIP likely arose via the duplication of a self-interacting protein (Safa et al. 2008). Exactly how caspase 8 and cFLIP coordinate the switch between apoptosis and necroptosis, and other specific targets, is not known, but an evolutionary analysis of cFLIP would likely shed light on the mechanism that regulates caspase 8 life-or-death roles. The closely related caspase 10 also interacts with cFLIP and plays a role in developing multiple myeloma, which is a malignant proliferation of plasma cells in the bone marrow (Lamy et al. 2013).

6.3.3.1 Caspase Polymorphisms and Cancer Prognosis

Several putative caspase polymorphisms are statistically significant markers for cancer prognosis. A relatively well-characterized six-nucleotide indel (insertion-deletion) polymorphism in the promoter region of CASP8 (rs3834129) is a predictor of mRNA expression levels (Sun et al. 2007; Kuhlmann et al. 2016). The allele-dose-dependent association between the CASP-8 -652 6N del allele and decreased caspase 8 mRNA expression is a negative prognostic marker in breast cancer where homozygous deletion carriers are at highest risk of death (Kuhlmann et al. 2016). One might suspect that a decrease in caspase 8 expression would decrease sensitivity to extrinsic apoptosis signaling due to the induced proximity model of activation. Patients with the CASP-8 -652 6N del allele, however, exhibit a decreased susceptibility to developing many cancers (Sun et al. 2007; Liamarkopoulos et al. 2011). The patients also exhibited a decreased risk of death compared to patients with the homozygous insertion genotype in a sample of Chinese gastric cancer (Gu et al. 2014) and oral squamous cell carcinoma (Tang et al. 2015). Biochemical analyses revealed that the presence of the CASP-8 -652 6N del allele in T lymphocytes lowers caspase 8 activation-induced cell death, and T lymphocytes are integral to our natural cancer defense mechanisms (Sun et al. 2007). Interestingly, the CASP-8 -652 6N del allele had no influence on colorectal cancer susceptibility in the UK population (Yin et al. 2010). An earlier meta-analysis of CASP8 polymorphisms determined that the CASP-8 -652 6N del polymorphism is an overall good prognostic marker (Yin et al. 2010); however, more recent data suggests that prognostic interpretation of a polymorphism will depend on the specific context in which it occurs (Park et al. 2016). Caspase 8 polymorphisms have been characterized for use as prognostic markers for overall survival (OS) in Chinese patients with advanced lung adenocarcinoma for platinum-based chemotherapy treatment (Liu et al. 2017). The clinical outcome will clearly depend on a variety of contextual factors, but directed research efforts can begin to improve the odds of successful treatment. An expansion of our knowledge surrounding the clinical outcomes associated with polymorphisms in cancer-related genes will progress us toward the goal of individualized cancer therapy.

6.4 The Caspase Story Is Incomplete

The decapitation of a *Hydra* leads to production of a new head, and planaria can regenerate whole new individuals from smaller body fragments. Even the human liver has the ability to recover from a 70% loss, which is compromised by knocking down caspases 3 and 7 (Li et al. 2010). Such discoveries have implicated apoptosis as the driving force behind tissue regeneration after traumatic injury (Fuchs and Steller 2011), which could pose a problem for cancer therapies that aim to induce apoptosis via caspase signaling. Evidence from studies of both initiator and executioner caspases show the promotion of cell regeneration through apoptosis-induced compensatory proliferation (AiP) (Bergmann and Steller 2010). The mechanism appears to be due to mitogen release from stressed or injured cells and suggests a

connection between signaling that leads to tissue regeneration after traumatic injury and cancer growth (Fuchs and Steller 2011). AiP occurs when caspase activity induces neighboring cells to replace dead and/or dying tissue, a phenomenon known as "compensatory proliferation." The result of insufficient apoptosis may exacerbate certain cancers through mitogen release and JNK signaling (Ryoo and Bergmann 2012). However, AiP is not always an observed outcome of caspase activity, and the circumstances that trigger AiP are currently unknown (Fogarty and Bergmann 2017). Contrary to our current understanding of caspase signaling, low levels of caspase 3 were shown to predict a favorable response to chemotherapy in advanced colorectal cancer, so caspase 3 inhibition is now being explored as a therapeutic approach for certain cancers (Flanagan et al. 2016).

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Emerging Epigenetic Targets and Their Implications in Cancer Therapy

7

Mohmmad Shoab Mansuri and Sonam Mehrotra

Abstract

Epigenetics refers to alterations in the chromatin that regulate gene expression without changing the nucleotide sequence of DNA. An "epigenetic code" constituted by DNA methylation and different posttranslational modifications of histone proteins is crucial for the regulation of gene expression status in various cells. Maintenance of cellular identity and differentiation in a specific developmental context is regulated through maintenance of the chromatin structure of the cells. This process involves both genetic and epigenetic mechanisms. There is emerging evidence that defects in epigenetic regulation are often observed in human cancer. The reversibility of epigenetic modifications and an improved understanding of epigenetic aberrations in the etiology of cancer promise identification of novel drug targets and development of epigenetic-based therapeutic avenues for cancer. Many such compounds that target chromatin-associated proteins and proteins involved in epigenetic regulation are currently under preclinical and clinical trials. In this chapter, we have summarized the discoveries of epigenetic-based therapies for cancer and highlighted the advancement in this field that provides a new future perspective.

Keywords

Epigenetic regulation · DNA methylation · Posttranslational histone modification and microRNA/miRNA

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7.1 Introduction

Cancer is a complex disease that was once thought to be initiated and driven largely by genetic alterations. Mutations causing loss of function of one or many tumor suppressor genes and/or a gain of function of an oncogene were considered as major causes of carcinogenesis (Hanahan and Weinberg 2011). Changes in the genetic landscape, however, are sometimes insufficient to explain the pervasive alterations in cellular function that culminate into hallmarks of cancer. Recent studies provide substantial evidence for the importance of epigenetic mechanisms during development, function of stem cells, and tumorigenesis.

Epigenetic modifications refer to variations in patterns of gene expression those occur during embryonic development or lifetime of an organism without causing any change in the DNA nucleotide sequences. The gene expression status in a cell is determined and regulated by its chromatin structure especially that of the genomic regions such as the promoters, enhancers, and insulators. These are further influenced by the presence or absence of the transcription factor proteins that bind to these regions. Dynamic and reversible regulation of chromatin is brought about by different epigenetic modifications which include DNA methylation, covalent modifications of histone proteins and their recognition by other protein modules (readers), alterations by ATP-dependent nucleosome remodeling, and expression of regulatory noncoding RNA molecules (Baylin and Jones 2016).

While alterations of epigenetic regulation can result in mutations in various genes, genetic mutations in epigenetic regulator can result in genome-wide changes in states of gene expression due to variations in DNA methylation patterns, nucleosome occupancy, or histone modifications. Thus, epigenetic changes impact concomitantly many different molecular processes, both in normal physiological and pathological states. The human cancer cells are widely believed to comprise of substantial alterations in their epigenetic regulatory mechanisms. Alternatively, epigenetic changes can also "prime" cells such that they promote cellular transformation upon a subsequent DNA mutation. Therefore, both epigenetic and genetic factors are intricately entwined with each other and not mutually exclusive (Fig. 7.1). To understand how altered gene silencing or expression culminates into cancer development, it is imperative to understand molecular mechanisms of epigenetic regulation. Therapies that aim to restore normal chromatin states by inhibiting different components involved in epigenetic regulation have been developed. The first to be developed therapeutics which targeted epigenetic mechanisms were compounds that inhibited histone-modifying enzymes such as histone deacetylases or DNA modifying enzymes such as DNA methyl transferases. The use of inhibitors to DNA methyltransferase and histone deacetylase proteins as drug-targeting epigenetic modifications has been further validated by their use in clinical trials. This further emphasizes the requirement to develop additional therapies that target epigenetic machinery.

In this chapter, we describe the association between epigenetic regulation of the chromatin structure involving changes in DNA methylation patterns, histone modifications, and expression of noncoding RNAs, as well as other epigenetic regulators with initiation and progression of cancer. We further focus toward current research involving biomarkers and emerging cancer therapies that target epigenetic regulators.

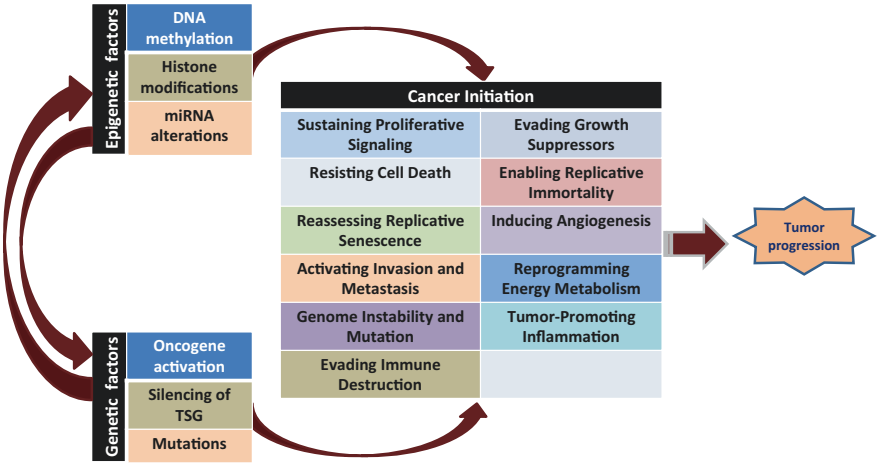

Fig. 7.1 Role of genetic and epigenetic aberrations in tumor initiation and progression

7.2 DNA Methylation

7.2.1 Regulation of Gene Expression by DNA Methylation

The first epigenetic alteration discovered in human cancer cells was changes in DNA methylation patterns of different genomic regions (Feinberg and Tycko 2004). A 5-methylcytosine arises due to the methylation of 5′-carbon of the cytosine residue (5mC) in a CpG dinucleotide repeat. These CpG islands are observed at many different genomic locations such as the promoter regions of the gene, inactive X-chromosome, centromeres, telomeres, and other repeat sequences (Saxonov et al. 2006). Multiple CpG islands present near promoter sites are methylated, which represses transcription and results in silencing of genes. These modifications are also crucial in maintaining genome stability as they are required for silencing of oncogenes and transposable elements (Sproul and Meehan 2013). Silencing of a gene may also be initiated by another mechanism, but it is often observed to be followed by hypermethylation of CpG islands (Bird 2002). Hypermethylation of CpG islands, especially those present in or near the promoter regions of several tumor suppressor genes, is crucial for initiation of several types of cancers. These CpG islands are also frequently present in promoters of regulatory RNAs, the roles of which are discussed later in the chapter. In addition, hypermethylation of CpG islands can also impact genes that are involved in regulation of cell cycle, DNA repair mechanisms, cell metabolism, cell-cell interaction, cell signaling pathways, and apoptosis, all of which are required for maintenance of genome integrity (Bird 2002). For example, in ovarian and breast cancers, the CpG islands located in the promoter region of *BRCA1* gene are observed to be hypermethylated in a tissue-specific manner. BRCA1 is known to be involved in homologous recombination-mediated, error-free type of repair of DNA double-strand breaks. Alterations in the expression of *BRCA1* have been frequently observed in many ovarian and breast

cancers and thus associated with the development of both these cancers. Therefore, *BRCA1* expression has emerged as a powerful biomarker for these two types of cancers (Esteller et al. 2000).

Epidemiological data suggests that CpG hypermethylation is more frequently observed with colorectal and other gastrointestinal cancers (Schnekenburger and Diederich 2012). In addition, in vivo, spontaneous somatic mutations due to conversions of methylcytosine to thymine by its deamination as well as germline mutations in the tumor suppressor gene *p53* are frequently observed at methyl CpG islands. These mutations have been shown to be strongly implicated in cancer initiation (Rideout 3rd et al. 1990).

The levels of DNA methylation are also influenced by several other factors that include aging, diet, and environment. Methyl group for DNA methylation can be obtained via folate and methionine synthesis pathways. As mammals are unable to synthesize folate and methionine, a diet low in these nutrients results in altered levels and patterns of DNA methylation which again increase susceptibility to cancer initiation (Moazzen et al. 2017). In addition, environmental factors such as exposure to arsenic and cadmium have also been shown to profoundly affect levels of DNA methylation. Arsenic has been shown to cause hypomethylation of oncogenes such as the *ras* gene, and cadmium has been shown to inactivate specific methyltransferase enzymes, which causes hypomethylation at a global level (Reichard and Puga 2010; Hossain et al. 2012; Kippler et al. 2012).

7.2.2 DNA Methylation and Allelic Imprinting

Gene imprinting refers to the process where only a single allele of a certain gene is expressed depending upon whether the allele is inherited from the mother or the father. DNA methylation plays an important role in the regulation of allelic imprinting. Most of the imprinted genes play critical roles during fetal development and growth. Approximately 100 imprinted genes have been identified in the human genome, the majority of which are functionally linked to signaling pathways involving insulin or insulin-like growth factor. Most of these have been observed to be affected in cancer (Uribe-Lewis et al. 2011). Examples of such imprinted genes include insulin-like growth factor 2 (*IGF2*), IGF2 receptor (*IGF2R*), growth factor-bound protein 10 (*GRB10*), stimulatory GTPase α (*GNAS*), and paternally expressed gene 3 (*PEG3*) and *H19* (Uribe-Lewis et al. 2011). The *IGF2* gene is specifically expressed from alleles of paternal origin. Regulation of DNA methylation is crucial in this process, such that aberrant methylation of the maternal allele of the IGF2 gene results in increased gene expression from its maternal copy. The increased expression of the *IGF2* genes results in elevation of IGF2 levels which has been linked to the increased predisposition of cancer development (Feinberg and Tycko 2004).

Study by the Cancer Genome Atlas (TCGA) systematically characterized alterations of imprinted genes in human cancer. This study suggested that in primary tumors, the expression of the major imprinted genes is reduced as compared to normal cells. The downregulation of these genes involved changes in DNA

methylation in imprinted domains such as the MEST, GNAS, and PEG3 domains which were observed in lung, ovarian, and mammary cancers. This study further highlights the correlation between the variation in the epigenetic regulation of such imprinted domains and their role as potential biomarkers in diagnosis of specific types of cancer (Kim et al. 2015).

7.2.3 DNA Methyltransferase Enzymes (DNMTs) and Their Inhibitors

DNA methylation is facilitated by specific enzymes that either mediate de novo methylation or maintain existing methylation states. Two methyltransferase enzymes, namely, DNMT3A and DNMT3B were discovered as those that establish de novo methylation profiles while DNMT1 was identified as the one required for maintaining established patterns of DNA methylation (Okano et al. 1999). In the past decade, compounds such as inhibitors of DNMT enzyme activity which have shown to result in hypomethylation of DNA at a global level in the cells have been developed. These have also been employed in clinical trials for acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) therapy. The first such compounds include cytidine analogs such as 5-aza-2′deoxycytidine/dacogen (DAC) and 5-azacytidine/vidaza (AZA). Azacitidine forms an irreversible covalent complex with DNMT1 which causes its degradation by a proteasome-mediated mechanism (Ghoshal et al. 2005). Low doses of DAC and AZA have been included along with treatment schedules for AML and MDS showing clear clinical benefits. Based on improvement of objective clinical response rates in patients treated with DAC and AZA in their specific phase III clinical trials, the US Food and Drug Administration (FDA) approved both of these drugs for treatment of MDS in 2004 and 2006, respectively (Ghoshal et al. 2005; Kantarjian et al. 2006; Kantarjian et al. 2007; Oki et al. 2007; Shen et al. 2010). Furthermore, both AZA and DAC were the first therapeutics based on inhibition of epigenetic regulation which were approved for cancer treatment.

AZA and DAC molecules, however, were prone to cytidine-deaminase-mediated destabilization. Therefore, more stable analogs were required. Analogs were modified to develop more stable forms. For example, SGI-110 comprises of deoxyguanosine dinucleotide (5′-DACpG-3′) that renders it resistant to cytidine-deaminase-mediated instability. Currently, SGI-110 is being tested in phase II clinical trials for treatment of AML, MDS, and chronic myelomonocytic leukemia (CMML) patients (Lavelle et al. 2010; Ning et al. 2016).

7.2.4 DNA Demethylation

Two studies from 2009 identified the ten-eleven translocation (TET) enzyme belonging to the family of DNA hydroxylases, which catalyzes the conversion of 5-methylacytosine (5mC) to 5-hydroxymethylcytosine (5hmC) (Tahiliani et al. 2009; Cimmino et al. 2011). These studies provided evidence for the role of 5hmC

and TET proteins activation and silencing of transcription. Further, genome-wide mapping of TET protein binding suggested that it had strong preference for regions enriched in 5mC and 5hmC modifications (Wu et al. 2011). TET proteins were also shown to alter the binding of several chromatin regulators by enhancing the binding of many methyl-CpG-binding proteins (Wu and Zhang 2011). Mutations in TET2 family of genes have been observed to be associated with several hematological malignancies and poor prognosis in a subset of AML patients (Saint-Martin et al. 2009; Delhommeau et al. 2009). These studies suggest that alterations in DNA demethylation contribute to both local and global effects in chromatin modeling which are associated with cancer development. So far, TET protein inhibitors have not been tested in clinical trials as therapeutics for cancer treatment.

7.3 Modifications of Histone Proteins

The pioneering work of Weintraub and Groudine led to the discovery of inactive and active regions in the chromatin which could be further distinguished based on their sensitivity toward nuclease and other such enzymes (Weintraub and Groudine 1976). This discovery sparked a keen enthusiasm in scientists to further understand the role of DNA histones on gene regulation. The DNA within the eukaryotic nucleus is packaged as chromatin that comprises of the nucleosome as its basic unit. Each nucleosomal subunit is composed of the four core histone proteins, namely, H2A, H2B, H3, and H4, forming an octamer. This histone octamer is wrapped around by a DNA segment of 147 base pairs. Each of the histone proteins consists of characteristic tail or side chain enriched with numerous basic lysine and arginine residues. These side chains can be extensively modified posttranslationally. These posttranslational modifications (PTMs) can mediate changes in chromatin structure which may be conducive to either repression or activation of target gene transcription. In addition to methylation and acetylation, histones may also be modified by other mechanisms which include sumoylation, ADP-ribosylation, ubiquitination, deamination, citrullination, formylation, *O*-GlcNAcylation, proline isomerization, propionylation, butyrylation, and crotonylation (Zhao and Garcia 2015, Huang et al. 2015). The functional consequences of these modifications depend upon the type of residues and specific modification. Thus, histones are not just DNA packaging molecules but central regulators of transcription that store epigenetic information through these posttranslational modification (PTMs) signature forming a "histone code." For example, the methylation of lysine 4 on histone 3 is closely associated with transcriptional modification, whereas methylation of lysine 9 or 27 on H3 is closely linked with repression of transcription. On the contrary, the acetylation of histone lysine is generally associated with activation of transcription. Aberrant histone modifications can therefore activate a large number of genomic loci that are repressed in a physiological state and vice versa. In the following section, we shall discuss the roles played by histone modifications in development of cancer and therapies based on inhibitors of histone-modifying enzymes.

7.3.1 Histone Acetylation

The transcriptionally active or the "open" chromatin confirmation is associated with comprising of lysine residues modified by N-acetylation. This process is critically regulated by the competing enzymatic activities of two special classes of proteins called histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Loidl 1994; Grunstein 1997; Cress and Seto 2000). The first enzymes identified to exhibit histone modifications were the HATs. In mammals, these can be broadly classified into three distinct families: (1) the Gcn5-related N-acetyltransferase (GNAT) family including Gcn5 and p300/CBP-associated factor (PCAF), (2) the MYST family comprising of monocytic leukemia zinc finger (MOZ) and TAT-interactive protein (Tip60), and (3) the p300/CBP family that consists of CREB-binding protein and p300 (Nair and Kumar 2012).

A number of examples of oncogenic mutations and chromosomal translocations which involve alterations in activities of various HATs such as p300 and CBP are observed in a broad range of hematological malignancies and solid tumors (Iyer et al. 2004; Wang et al. 2005; Huntly et al. 2004). In addition to histones proteins, several nonhistone proteins including both oncogenic and tumor suppressor proteins, such as PTEN, MYC, and P53, respectively, undergo dynamic acetylation (Choudhary et al. 2009). Therefore, acetylation of histones as well as other proteins is implicated in carcinogenesis which may serve as potential targets for developing novel therapies.

Transcriptionally repressive or closed chromatin states have been shown to be created by deacetylation of histones. Several cellular functions involve the activity of HDACs. The aberrant expression of HDACs can result in constitutive repression of tumor suppressor genes causing cancer development. The HDACs so far have been classified into four families: Classes I, II, III, and IV. Classes I, II, and IV comprise Zn^{2+}-dependent HDACs, whereas members of the Class III family are NAD dependent and also called sirtuins (Seto and Yoshida 2014). Although mutations in HDACs are not frequently observed in cancer, alterations in activity of different HDACs are frequently observed in several malignancies. Several pan-HDAC inhibitors have been developed that have validated in various clinical trials on patients of T-cell lymphoma. Two of these HDAC inhibitors, namely, vorinostat and romidepsin, have recently been approved by the FDA for treatment of patients with cutaneous T-cell lymphoma (Glozak and Seto 2007). HDAC inhibitors can aid in re-establishing acetylation patterns as observed in tumors with mutations in HAT such as EP300. Currently, inhibitors of different HDACs such as HDAC1, HDAC2, HDAC3, and HDAC8 are being developed and other being tested in preclinical studies.

Chromosomal translocations observed in cancer often show defects in recruitment of protein complexes containing HDAC enzymes to different promoter regions. For example, retinoic acid receptor α (RARα) translocation is observed in promyelocytic leukemia (PML). This translocation causes aberrant recruitment of the N-CoR/HDAC repressor complexes and further repression of many RAR target genes (Minucci and Pelicci 2006). In addition, HDAC/sirtuin members of Class III family possess a distinct mechanism of catalysis. These include deacetylation on both histone and

nonhistone substrates as well as modifications by ADP-ribosylation and desuccinylation of various proteins (Seto and Yoshida 2014). SIRT1, a Class III HDAC, is overexpressed by several tumors and reduced in others. This suggests that these proteins are observed to be over-expressed or reduced in various tumors. Combination of Class I/II HDACs inhibitors such as vorinostat and Class III HDAC inhibitors cambinol is being tested in neuroblastoma animal models (Lautz et al. 2012).

Due to relatively low substrate specificity of HDACs, an individual HDAC enzyme catalyzes deacetylation on more than one histone sites. Their functions are also context dependent. Their ability to modify histone and nonhistone proteins makes it difficult to elucidate the role of specific HDACs in tumorigenesis. Small molecule inhibitors for specific HDAC isoforms are being developed which are expected to emerge as HDAC modulators of higher therapeutic value.

7.3.2 Histone Methylation

KDM1 was the first demethylase enzyme to be discovered. It provided important insights into the significance of histone methylation in regulation of gene expression. The lysine, arginine, and histidine side chains of histone proteins can be modified to multiple methylation states, which conjures extensive functional complexity. Tight regulation of histone methylation states is brought about by competing activities of various methyltransferase and demethylase enzymes that function in coordination to add or remove specific methyl groups. The methylation patterns are critical for regulation of gene expression, cell differentiation, stem cell functions, and genomic stability.

The lysine residues are one of the best characterized sites that are modified during mono-, di-, or trimethylation of the histone proteins. These include but not limited to H3K4, H3K9, H3K27, H3K36, H3K79, and H4K20 methylation. Among these specific sites of lysine methylation, H3K4, H3K36, and H3K79 are associated with transcriptionally active chromatin regions, whereas modified H3K9, H3K27, and H4K20 are linked with transcriptionally repressed chromatin regions or heterochromatin. Different methylation patterns are observed on specific genomic regions. For example, the transcription start site is enriched with H3K4me2/3 marks, while H3K4me1 is observed on active enhancer regions. Thus, methylation patterns are crucial factors also that define chromosome boundaries in the eukaryotic genome. Deregulation of methylation signatures brings about global variations in chromatin structure and profound alterations in states of gene expression. Early stages of cancer have been shown to be correlated with deregulation of H3K4 and H3K27 methylation signatures which are frequently observed in different types of tumors. Unlike HATs, histone lysine methyltransferases are highly substrate specificity that are target methylation modifications on specific lysine residues. Hence, inhibition of specific methyltransferase enzymes can be used to target methyl modification of specific lysine residues which may be altered in a particular cancer. Therefore, inhibitors of lysine methyltransferase enzymes serve as powerful targets for development of cancer therapy.

7.3.3 Targeting Histone Lysine Methyltransferase Enzymes

Most of the histone methyltransferase enzymes consist of conserved **S**u(var)3-9, **E**nhancer-of-Zeste, and **T**rithorax (SET) domains that confer methyltransferase enzyme activity. Dysregulation of a family of lysine methyltransferases (KMTs) by either loss of function mutations or translocations is implicated in many cancers including MLL. These enzymes specifically methylate H3K4. The MLL fusion proteins may not retain the SET domain; however, they possess the DNA-binding ability which can inappropriately alter the expression of critical genes such as Hox genes.

H3K27 di- or trimethylation modifications are catalyzed by EZH2 and have been linked with closed or repressed chromatin states. Alterations of H3K27 methylation profiles are observed in several cancers including those of breast, prostate, and bladder. The Enhancer-of-Zeste 2 (EZH2) is the catalytic component of the Polycomb Repressive Complex 2 (PRC2) complex. Mutations in the SET domain of EZH2 that alter its substrate preference and increase its catalytic activity are found to be oncogenic mutations implicated in several cancers. Diffused large B-cell lymphoma tumors harboring EZH2 mutations are extremely sensitive to EZH2 inhibitors (Morin et al. 2010). In addition, mutations conferring loss of function of EZH2 gene have also been observed in T-cell acute lymphoma and myeloid malignancies. These are also associated with poor prognosis in these patients (Ernst et al. 2010). Small molecule inhibitors targeting EZH2 provide great promise for their clinical translation. Preclinical trials using EZH2 inhibitor DZNeP in combination with pan-HDAC inhibitor panobinostat induced apoptosis only in AML cells but not in bone marrow progenitor cells (Fiskus et al. 2009, Wang et al. 2009). Additional EZH2 inhibitors which are being tested include EI1 and GSK126. EI1 directly binds to EZH2, thus inhibiting its enzymatic activity. The proliferation of diffused large B-cell lymphoma cell lines and xenografts containing EZH2 mutations are inhibited by GSK126. Further clinical I/II trials for EZH2 inhibitor EPZ-6438 have been initiated for patients with B-cell lymphomas and advanced solid tumors (Fiskus et al. 2009). EPZ-6438 is the first drug that is based on the inhibition of EZH2 activity that has been effectively observed in solid tumors.

Closed chromatin states and repression of gene expression are generally associated with H3K9me2 and H3K9me3 histone methylation signatures, respectively. Alterations of H3K9 methylation marks observed in cancer and candidate drugs targets based on the inhibition of histone methyltransferase enzymes have been developed. BIX-01294 and 3-deazaneplanocin are examples of H3K9 methyltransferase inhibitors that effectively reduce proliferation and induced apoptosis of neuroblastoma cells in culture (Vedadi et al. 2011). Mithramycin diminishes the expression of *SETDB1* gene that encodes a H3K9-specific methyltransferase. Mithramycin is a clinically approved antitumor therapeutic (Ceol et al. 2011).

7.3.4 Therapeutics Based on Inhibition of Histone Demethylases

The identification of two types of histone demethylases overturned the opinion that histone methylation was non-dynamic and a highly stable modification. These

classes of demethylases include JMJC-domain-containing lysine demethylase family and lysine-specific demethylase 1 (LSD1/KDM1) family (Mosammaparast and Shi 2010). Many genes encoding for histone demethylases are overexpressed in different tumors, and their copy number is also genetically amplified in cancer cells. These characteristics have resulted in studies that assess HMTs as putative candidates for development of high-affinity and specific inhibitors of HMT for cancer treatment (Mosammaparast and Shi 2010).

7.3.4.1 The Family of Lysine-Specific Demethylases

The lysine-specific demethylase family comprises only two histone demethylases: LSD1 (KDM1A) and LSD2 (KDM1B). These enzymes contain an oxidase-like domain which confers their catalytic activity. The mechanism of removing a methyl group from lysine side chains of histone is an oxidation reaction dependent upon cofactor FAD (Shi et al. 2004). Enzymes of the LSD family catalyze demethylation of only mono- and dimethylated lysines but not trimethylated lysines. Overexpression of LSD1 protein is observed in cancers of the prostate, bladder, colon, and mammary tissue. Therefore, LSD1 protein is being assessed as biomarker for these tumors and a potential drug target (Toyokawa et al. 2011; Hayami et al. 2011).

More potent and specific inhibitors of LSD1 enzymes are also being developed which include derivatives of tranylcypromine. These have been shown to be more effective in the treatment of refractory acute leukemia (Schenk et al. 2012). One such potential therapeutic called ORY-1001 has been developed and tested by the biotech company Oryzon Genomics. Polyamines are another group of molecules that inhibit LSD1 but lack target selectivity (Wang et al. 2011). Therefore, other than tranylcypromine derivative, no other promising compounds that specifically target LSD have been published.

7.3.4.2 The Family of Demethylases Containing the JMJC Domain

This family of histone demethylases contains the characteristic JMJC domain. They require two cofactors, iron and 2-oxoglutarate (Kooistra and Helin 2012). JMJC demethylases remove methyl groups from mono-, di-, and trimethylated lysines. Many genes coding for the JMJD2 enzymes are overexpressed and/or genetically amplified in tumors of mammary tissue, squamous cell carcinoma, and medulloblastoma (Liu et al. 2009; Ehrbrecht et al. 2006). In addition, expression of JARID1 family enzymes is also altered in bladder and breast cancers and in some types of leukemia (Nguyen et al. 2011). Due to a strong association between JMJC alterations with cancer, the therapeutic potential of compounds that inhibit the enzymatic activity of JMJC proteins is being assessed.

Other potent and selective inhibitors which exhibit suitable properties such as cell permeability include 8-hydroxyquinolines and pyridine hydrazones (Chang et al. 2011). In addition, inhibitors of the JMJD3 subfamily that competitively bind to 2-oxoglutarate cofactor have also been developed, which exhibited encouraging results in preclinical trials (Heinemann et al. 2014).

7.3.5 Targeting Other Atypical Histone Modifications

7.3.5.1 Histone Phosphorylation and Ubiquitylation

In addition to histone modifications by methylation and acetylation, histones are also modified by phosphorylation and ubiquitination. Phosphorylation of histone H3S10 is observed during mitosis and cytokinesis and requires Aurora-B protein. Inhibitors of Aurora kinase such as ZM447439, Hesperadin, and VX-680 are currently being tested in preclinical studies (Hirota et al. 2005).

Histone phosphorylation of H3T41p is catalyzed by Janus-associated kinase (JAK2), a type of cytoplasmic tyrosine kinase. Inhibitors of JAK2 tyrosine kinase known as INCB018424 have exhibited clinical benefits in patients with myelofibrosis. Lestaurtinib and pacritinib are other JAK2 inhibitors that are under clinical trials for treatment of myelogenous leukemia and other advanced myeloid malignancies (Verstovsek et al. 2010; Shabbir and Stuart 2010).

Histone ubiquitylation is mediated through PRC1 and plays crucial roles in gene silencing. Inhibitors of PRC1-mediated H2A ubiquitylation known as PRT4165 exhibit inhibition of E3 ubiquitin ligase activity and is being further investigated for therapeutic potential (Ismail et al. 2013).

7.3.5.2 Arginine Citrullination

Like lysine residues, histone arginine residues can also be methylated. In addition, both unmodified or monomethylated arginine residues can also undergo a hydrolysis reaction to form citrulline. This process is known as deamination or citrullination. This reaction is enzymatically catalyzed by the enzymes of the PAD family of proteins which also remove the positive charge from the arginine side chain. Of the members of the PAD family, only PAD4 deiminates citrullinate arginine residues on histones H3, H2A, H4, and H1 (Wang et al. 2004; Christophorou et al. 2014). Citrullination of histone is a tightly regulated process that is involved in the decondensation of chromatin and observed only in naïve pluripotent cells such as inner cell mass cells and embryonic stem cells (Christophorou et al. 2014).

Overexpression of PAD4 has been observed in many tumor tissues such as ovarian, hepatocellular, breast, and non-small-cell lung carcinomas [NSCLC] as well as cell lines. (Chang et al. 2009). The role of PAD4 alterations in tumorigenesis remains to be understood. A keen interest in developing inhibitors for PAD family members, especially PAD2 and PAD4, provides newer therapeutic avenues in cancer and inflammatory diseases. Currently, studies involving PAD inhibitors are in early phases of preclinical trials which will require more time to elucidate their therapeutic potential.

7.3.5.3 Histone Lysine ADP-Ribosylation

A relatively rare phenomenon observed in <1% of all histone proteins involves their modification by ADP-ribosylation of lysine residues. These modifications, however, are particularly observed at sites of single-strand DNA breaks (Boulikas 1989).

These modifications are catalyzed by some NAD^+-dependent poly(ADP-ribose) polymerases (PARPs), which ADP-ribosylate both histone and nonhistone proteins. Among these enzymes, PARP1, also known as ARTD1, is one of the most studied and characterized histone ADP-ribosylating enzyme. This enzyme is activated in response to environmental stress or DNA damage and is capable of modifying all core histone at specific positions (Messner et al. 2010). These PTMs are associated with decondensation of the chromatin and further involved in recruitment of DNA repair machinery. The acetylation of H4K16 inhibits ADP-ribosylation by PARP1 suggesting a cross talk between different lysine modifications such as ADP-ribosylation and acetylation signatures (Messner et al. 2010). The biological functions of histone ADP-ribosylation and specific PARP enzymes in regulation of chromatin structure are still not clearly understood. Although PARP1 inhibitors such as olaparib are used in treatment of ovarian and breast cancers with mutated BRCA1 and BRCA2 genes, the use of PARP inhibitors in combination with histone ADP-ribosylation inhibitors still requires approval.

7.3.5.4 Histone O-GlcNAcylation

Recent studies reveal that modifications of core histones by *O*-GlcNAcylation can also occur at specific Ser and Thr residues by the addition of β-*N*-acetylglucosamine. These PTMs are observed during heat shock and mitosis. The known sites of *O*-GlcNAcylation can also undergo phosphorylation suggesting that *O*-GlcNAcylation may interfere with other modifications. Therefore, overexpression of *O*-GlcNAc transferase (OGT) enzyme is observed with an increase in chromatin condensation, suggesting its role in maintenance of chromatin status and potentially genome integrity (Sakabe et al. 2010; Wang et al. 2010).

7.3.6 Targeting Epigenetic Readers

Enzymes such as histone methyltransferases and HATs which modify histones by addition of specific chemical groups are known as "writers," and enzymes that remove these modifications, such as histone demethylases and HDACs, are known as erasers. There is another class of proteins that specifically recognizes these specific modifications and in response initiates specific signaling cascades. These proteins are known as "readers." This class of proteins includes many chromatin-binding proteins such as the malignant brain tumor (MBT) protein, bromodomain and extra-terminal (BET) proteins, and plant homeodomain (PHD) finger proteins(Taverna et al. 2007). Each family of chromatin reader proteins possesses specific domain or a variant which is capable of altering their preferred binding substrate. For example, the PHD finger domain in the protein ING2 binds to di- and trimethylated lysines, while the PHD finger domains of DNMT3L and BHC80 proteins prefer binding to unmethylated lysine residues. If the lysine residue undergoes acetylation, a different type of binding site is generated. This modification in turn recruits different class of readers such as proteins possessing bromodomain like acetyl-lysine-binding motifs. Thus, the structure of reader proteins present at specific locations on the chromatin

depends upon the profile of surrounding histone modifications (Ruthenburg et al. 2007).

7.3.6.1 Inhibitors of Bromodomain and Extraterminal (BET) Proteins

Bromodomains are highly conserved motifs present in reader proteins that identify and bind to acetylated lysine residues on histone proteins. The binding of these proteins to acetylated histones forms a scaffold-like structure required for the recruitment and assembly of multiprotein complexes that facilitate DNA template-dependent physiologically important processes. More than 50 bromodomain proteins have been identified in humans, and based on their sequence homology, these are classified into nine subfamilies (Filippakopoulos and Knapp 2012; Filippakopoulos et al. 2012).

The BET family of proteins contains a tandem bromodomain at their N-terminal. There are four family members: BRD2, BRD3, BRD4, and BRDT. These proteins have crucial roles in regulation of transcription. For example, BRD4 is associated with the active form of transcription elongation factor b (P-TEFb) which regulates the processivity of RNA polymerase II, resulting in the expression of growth-promoting genes. These proteins, therefore, also regulate cell growth. BET proteins often function as components of large multiprotein complexes that are involved in many cellular processes such as transcription, chromatin remodeling, and DNA replication and repair (Yang et al. 2005). Therefore, alterations in the levels of BET proteins, specifically BRD3 and BRD4, are correlated with several cancers such B-cell lymphoma, NUT midline carcinoma, and AML (Greenwald et al. 2004; French 2010). These discoveries have encouraged the elucidation of therapeutic candidates that target BET proteins. A small molecule BET inhibitor, JQ1, was included in a study involving a panel prostrate cancel cell lines. This study revealed that JQ1 was capable of inducing apoptosis and cell cycle arrest in cells with activated androgen receptor signaling. JQ1 also targets MYC gene, the transcription of which is reduced by JQ1. BET bromodomain inhibitors I-BET151 and I-BET762 have been shown to reduce the expression of important oncogenes such as MYC in multiple myeloma and undergoing clinical trials for the treatment of hematological malignancies (Asangani et al. 2014; Chaidos et al. 2014). A novel competitive antagonist of BET proteins called dBET1 has also been developed which is capable of inducing highly specific BET protein degradation and is currently being studied using animal models (Winter et al. 2015).

7.3.6.2 Inhibitors of MBT Proteins

Proteins that recognize methylated lysine residues also include the MBT, Tudor, and chromodomain-containing proteins (Herold et al. 2011). About 30 Tudor-domain-containing proteins have been identified in mammals till date of which several are altered and associated with different cancers. Tudor-domain-containing protein 1 (TDRD1) is observed to be overexpressed in prostate cancer, whereas L3MBTL3, an MBT protein, is found to be underexpressed in several hematological malignancies. Inhibitors of L3MBTL3 proteins UNC1215 and UNC1679 have been developed and currently being tested in preclinical trials for their therapeutic potential in treatment of hematological malignancies (James et al. 2013).

7.4 Expression of Noncoding miRNAs and Cancer

It has become evident in the recent years that the non-protein-coding regions of the genome do not constitute counterfeit transcriptional noise but play key biological functions in normal cellular physiology as well as various diseases, particularly cancer. ncRNAs include both small ncRNAs (< 200 nucleotides) and long ncRNAs. Further, small ncRNAs can be divided into endogenous small-interfering RNAs (endo-siRNAs), microRNAs (miRNAs), small nucleolar RNAs (snoRNAs), and PIWI-interacting RNAs (piRNAs). Due to their crucial roles in many biological processes, miRNAs have attracted researchers to a great extent. miRNAs are naturally occurring, endogenous, small ncRNAs, which are highly conserved throughout evolution. Mature miRNAs comprise 18–22 nucleotide (nt)-long molecules that are formed by the cleavage of 70–100 nt hairpin pre-miRNA precursor molecules (Bartel 2004). Single-stranded miRNAs block translation or degradation of target mRNAs by binding to the 3′-untranslated region (3′UTR) of target mRNAs mediated by partial sequence complementarity (Bartel, 2004). miRNAs target more than 60% of protein-coding genes, and >45,000 miRNA target sites are conserved in 3′UTRs of human genes (Friedman and Jones 2009). A single miRNA may target many mRNAs, and a single mRNA may also be targeted by a number of miRNAs. Therefore, anomalous miRNA expression can affect the expression of different genes which eventually can disrupt the regulation of several biological processes. The abnormal expression profiles of miRNA are thus frequently observed in several disease states, including human cancers (Pasquinelli et al. 2005; Calin and Croce 2006; Esquela-Kerscher and Slack 2006; Garzon et al. 2009a). Altered miRNA expression profiles have been broadly accepted as one of the hallmarks of cancer. In addition, genome-wide association studies (GWAS) have provided substantial evidence that ~50% of miRNAs are located at fragile sites of chromosomes, genomic regions prone to loss of heterozygosity, or other regions associated with cancer (Calin et al. 2004). These observations further implicate the role of miRNAs in carcinogenesis.

Further studies indicate that miRNAs may play dual role as a new oncogene or a tumor suppressor gene (TSGs) that may be critical in initiation of carcinogenesis. For example, miR-21 is universally upregulated in many cancers where it acts as an oncogene and suppresses the expression of downstream target genes that regulate cell proliferation, death, or differentiation (Meng et al. 2007; Frankel et al. 2008; Dillhoff et al. 2008). In contrast, miR-34 family (miR-34a and miR-34b/c) genes act as tumor suppressor genes and are regulated by p53, which in turn is also universally downregulated in different types of cancers. For instance, reduced expression of miR-34a and miR-34c has been reported in certain breast cancers exhibiting lymph node metastasis, while repression of Fos-related antigen 1 (Fra-1) oncogene mediated by the activation of miR-34a/c is shown to restrain the metastasis and invasion of breast cancer (Yang et al. 2013). Several instances where miRNAs act as oncogenes have also been observed. This includes downregulation of miR-25 expression in colon cancer cells where it inhibits their growth and migration by directly targeting Smad7 (Li et al. 2013). miR-25 expression has also been observed to be upregulated in esophageal squamous cell carcinoma (ESCC) where it induces their abilities

of invasion and migration by reducing the expression of E-cadherin (CDH1) (Xu et al. 2012). These findings suggest that regulation of several miRNAs is tissue specific, and consequences of the expression profile are context dependent.

There are increasing evidences that demonstrate the vital roles played by miRNAs in many cellular processes associated with cell proliferation, differentiation, metastasis, invasion, apoptosis, as well as development. Therefore, it is essential to elucidate and understand the mechanisms which result in altered expression of miRNAs as observed in cancer. Various studies have investigated mechanisms that regulate miRNA expression which also include regulation by epigenetic modifications. In addition, epi-miRNAs, a subgroup of miRNAs, have recently been discovered in direct or indirect target enzymes and molecules involved in regulation of epigenetic modifications.

7.4.1 DNA Methylation and miRNAs

miRNAs can regulate DNA methylation mainly by two mechanisms: (1) by modulating DNMTs and (2) by modulating methylation-related proteins (Wang et al. 2018). For instance, miR-29 family members that are predicted to target DNMT1, DNMT3A, and DNMT3B are often observed to be downregulated in several cancers. The expression of miR-29 has been inversely correlated with expression of DNMT3A and DNMT3B genes in lung cancer. In fact, overexpression of miR-29 is linked with decreased DNA methylation in acute myeloid leukemia and lung cancer and reactivation of tumor suppressor genes (Cattaneo et al. 2015; Fabbri et al. 2007; Garzon et al. 2009a). DNMT1 is also targeted by several other miRNAs such as miR-148a-3p, which reduce DNA methylation in laryngeal squamous cell carcinoma (Wu et al. 2016). miRNAs miR-148a and miR-152 also decreased the expression of DNMT1 protein and simultaneously increased the expression of Rassf1a and p16INK4a, thereby resulting in reduced cell proliferation in malignant cholangiocytes (Braconi et al. 2010). Both miR-124 and miR-506 target DNMT3b and DNMT1 and are downregulated in colorectal cancer cells (Chen et al. 2015). miR-101 functions as a tumor suppressor gene in glioma cells where it targets Kruppel-like factor 6 (Yao et al. 2015). In addition, miR-101 also directly targets DNMT3a in astrocytoma cells and suppresses the expression of PRDM16 by modifying the methylation pattern of the PRDM16 promoter. PRDM16 has been shown to be hypomethylated and overexpressed in astrocytoma patients. It has also been demonstrated that the hypomethylation of PRDM16 promoter can be used to predict prognosis for astrocytoma (Lei et al. 2016).

7.4.2 miRNAs and Histone Modifications

An interesting cross talk takes place between miRNA-mediated epigenetic modifications and key histone modifications. Key targets of miR-101 include PRC2 and EZH2 proteins which are involved in gene silencing. The major mechanism involves

PRC2 activity which is altered due to downregulated miR-101 levels which in turn increase EZH2 levels (Varambally et al. 2008). Further, reactivation of miR-101 suppresses EZH2 expression, thus inhibiting cell proliferation and invasiveness (Cao et al. 2010).

Interestingly, downregulation of Dicer, a key protein involved in processing of miRNA, leads to elevated expression of PRC components including several PRC1 and PRC2 proteins. As a whole, PRC complex is controlled by miRNA through multiple targets (Cohen et al. 2009). After H3K27 methylation, both PRC1 and PRC2 complex proteins are recruited to the chromatin and are involved in sustained stable gene silencing mediated through ubiquitinylation of histone H2A. Expression of miR-203, 200b/c, miR-181a/b, and miR-200b/c decreases the levels of global ubiquityl-H2A, which in turn regulates chromatin states (Cohen et al. 2009). These findings have demonstrated the multifaceted cross talk of epigenetic components in controlling gene expression states.

miRNAs have also been shown to regulate the expression of HDACs. miR-499a directly targets HDAC1 in PC-3 cells, and it is involved in cell cycle regulation (Noonan et al. 2009). The global- versus locus-specific effects of HDACs are emerging which are also involved in the repression of HDAC1 via miR-449a (Noonan et al. 2009). These studies suggest the involvement of combinatorial epigenetic regulation of key growth regulators. miRNAs have also been shown to be involved in the regulation of HATs. p300/CBP-associated factor (PCAF), which possesses HAT activity, is targeted by miR-17-5p that further regulates the androgen receptor activity in prostate-derived cell lines (Gong et al. 2012). Overall, these findings suggest that miRNAs play a key role in fine-tuning both the activation and silencing of epigenetic marks (Table 7.1).

7.4.3 Cross Talk Between DNA Methylation, Histone Modifications, and miRNAs Expression

DNA methylation, histone modifications, and expression of miRNAs constitute three main epigenetic modifications that also interact and influence each other (Fig. 7.2). For instance, miRNA expression can be governed by methylation in their respective promoter regions, while miRNAs can directly target and regulate the expression of DNMTs and other chromatin remodeling enzymes (Duursma et al. 2008; Saito and Jones 2006). In contrast, DNMTs silence miRNAs, for example, silencing of miR-26a in prostate cancer induces the expression of its target EZH2 and alteration of global DNA methylation status (Borno et al. 2012). Promoter CpG island hypermethylation silences miR-137 which targets lysine-specific demethylase 1 (LSD1) in colorectal adenomas (Balaguer et al. 2010). Stabilization of DNMT1 by LSD1 leads to a positive feedback loop between them. In addition to this cross talk between histone methylation and DNA methylation, interactions between histone deacetylation and DNA methylation marks also occur which is mediated via miRNA involving miR-29 and miR-1. miR-1 was found to be

Table 7.1 miRNAs regulating DNA methylation in human cancers

miRNA/ epi-miRNA	Target/s	Cancer type/cell
miR29b	DNMT1, DNMT3a, DNMT3b	Acute myeloid leukemia, lung cancer, nasopharyngeal carcinoma
miR-148a, miR-152	DNMT1	Cholangiocarcinoma
miR-124, miR-506	DNMT1, DNMT3a	Colorectal cancer
miR-155-5p	DNMT1	
MG98	DNMT1	Renal cell carcinoma
miR-140	DNMT1	Hepatocellular carcinomas
miR-143	DNMT3a	Colorectal cancer
miR-221	DNMT3b	Breast cancer
miR-145	DNMT3b	Prostate cancer
miR-212	MeCP2	Gastric cancer
miR-373	MBD2	Hilar cholangiocarcinoma

downregulated in human hepatocellular carcinoma (HCC) cells due to hypermethylation by DNMT1, thus increasing HDAC4 expression (Datta et al. 2008). Similarly, miR-29 is silenced by HDACs in acute myeloid leukemia (AML) while consecutively increasing the expression of its target gene DNMT3 (Garzon et al. 2009a, b). These studies provide evidence suggesting that the interplay between different epigenetic regulators can be intervened by miRNAs.

In CaP cell lines, HDAC1, HDAC2, HDAC4, and DNMT1 are targeted by miR-34b. On the other hand, DNA methylation silences the miR-34b expression. Ectopic expression of miR-34b results in the demethylation of 5′ upstream sequence of its own gene and further causes trimethylation of histone H3 lysine 4 (H3K4me3), thus enriching active chromatin (Majid et al. 2013). These studies provide examples of interplay between miRNA and epigenetic modifications of histones and DNA.

7.4.4 Targeting miRNA Expression for Cancer Therapy

The interactions of miRNAs with epigenetic regulators and their recurrent alterations suggest their potential as novel biomarkers and therapeutic targets in treatment of cancer. For their translation into clinical applications of miRNAs in cancer therapy, following approaches are being developed: (1) use of miRNA antagonists to inhibit oncogenic miRNAs (anti-miRs or antagomiRs) and (2) use of synthetic miRNA mimics or stable vector-based miRNA transfection to introduce miRNA with tumor suppressor functions (Azmi et al. 2011). In addition, an in-depth knowledge of how miRNAs are epigenetically regulated will pave way to a newer therapeutic approach based on use of molecules that can fine-tune the expression of both oncogenic and tumor suppressor miRNAs. These will be very relevant in cancer treatment.

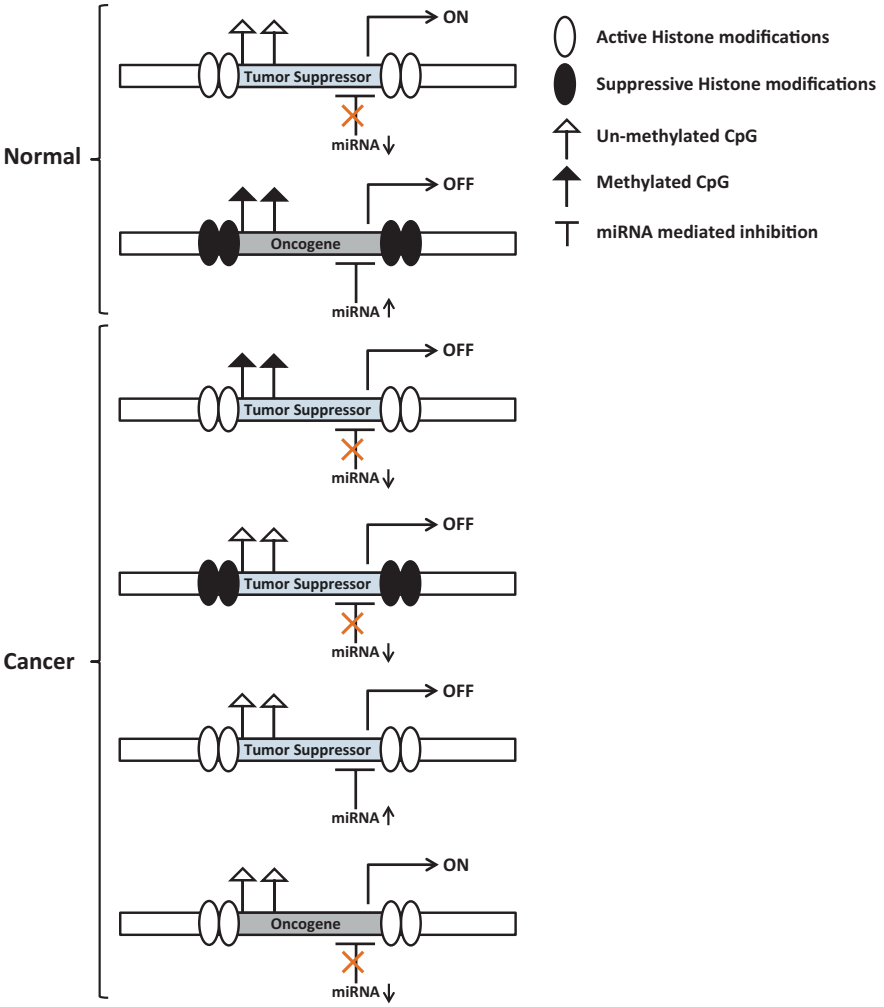

Fig. 7.2 Epigenetic regulation gene expression in cancer. Example of a tumor suppressor gene (TSG) and an oncogene with their respective CpG islands and target miRNAs. In normal cells, the CpG islands in the TSG are unmethylated. The chromatin is associated with active histone modifications, and target miRNA is silenced for TSG. In cancer cells, CpG island hypermethylation, repressive histone marks, and upregulated target miRNA expression lead to epigenetic silencing of the TSG. Alternatively, oncogenes are silenced through repressive histone modifications, CpG island hypermethylation, and upregulated target miRNA expression in normal cells, which are reversed in cancer

The altered miRNAs observed in human cancers which have been shown to be successfully regulated using epigenetic drugs are listed in Table 7.2. These agents can either suppress the oncogenic miRNAs which are upregulated in cancer cells or induce the reactivation of silenced tumor suppressor miRNAs. Often, it is observed that miRNAs are regulated by numerous epigenetic factors and, therefore, inhibition

Table 7.2 Epigenetic drugs that target miRNAs in human cancers

miRNAs	Target protein	Cancer type	Epigenetic drugs	References
miR-1-1	FOXP1, MET, HDAC4	Hepatocellular carcinoma	5-AzaC	Datta et al., (2008)
miR-17-92 Cluster	PTEN, BCL2L11, CDKN1A	Colorectal cancer	SAHA or TSA	Humphreys et al. (2013)
miR-21	PDCD4, TPM1, and MARCKS	Prostate cancer	5-Aza-CdR	Hulf et al. (2011)
miR-15a/ miR-16	BCL-2 and MCL-1	Chronic lymphocytic leukemia	LBH589/ SAHA	Sampath et al. (2012)
miR-29	Mcl-1, DNMT3A, DNMT3B, SP1, Tcl-1, CDK6, and IGR1F	Aggressive B-cell lymphomas	DZNep + SAHA	Zhang et al. (2012)
miR-31	SRC, RAB27a, NIK and MET, RhoA, and WAVE3	Melanoma	5-Aza-CdR/ DZNep	Asangani et al. (2012)
miR-34	C-MYC, CDK6	Lung cancer, melanomas, prostate cancer	5-Aza-CdR	Lodygin et al. (2008)
miR-125b	PIGF	Hepatocellular carcinoma	5-Aza-CdR	Alpini et al. (2011)
miR-126	VEGFA, PIK3R2	Bladder cancer	5-Aza-CdR + PBA	Saito et al. (2009)
miR-127	BCL6	Bladder cancer and clear cell renal cell carcinomas	5-Aza-CdR + PBA/TSA	Saito and Jones (2006)
miR-132	HB-EGF, TALIN2	Prostate cancer	5-Aza-CdR or 5-Aza-CdR + TSA	Formosa et al. (2013)
miR-141, miR-514	HGF, CDC42, RBX1, SLC2A1	Clear cell renal cell carcinomas	5-Aza-CdR + TSA	Wotschofsky et al. (2013)
miR-145	BNIP3, TNFSF10, PAK7	Prostate cancer and clear cell renal cell carcinomas	5-Aza-CdR or 5-Aza-CdR + TSA	Wotschofsky et al. (2013) and Zaman et al. (2010)
miR-181a,b, miR-200a,b,c, and miR-203	BMI1 and RING2	Prostate cancer	DZNep/5-Aza-CdR/ SAHA	Cao et al. (2010)
miR-193b	ETS1, CCND1, PLAU	Prostate cancer	5-Aza-CdR + TSA	Rauhala et al. (2010)
miR-205	SIP1, ZEP, and BCL-w	Prostate cancer	5-Aza-CdR	Hulf et al. (2011)

(continued)

Table 7.2 (continued)

miRNAs	Target protein	Cancer type	Epigenetic drugs	References
miR-224	API-5	Hepatocellular carcinoma	C646	Wang et al. (2012)
miR-335	SOX4, Rb1	Hepatocellular carcinoma	5-Aza-CdR + TSA	Dohi et al. (2013)
miR-370	IRS-1	Oral squamous cell carcinoma	5-Aza-CdR	Chang et al. (2013)
miR-373	MBD2	Hilar cholangiocarcinoma	5-Aza-CdR or 5-Aza-CdR + TSA	Chen et al. (2011)
miR-449	CDK6, CDC25A, c-MET	Breast cancer	DZNep + TSA	Yang et al. (2009)

DNMT inhibitors: 5-azacytidine (5-AzaC), 5-aza-2′-deoxycytidine (5-Aza-CdR), zebularine
HDAC inhibitors: suberoylanilide hydroxamic acid (SAHA), trichostatin A (TSA), panobinostat (LBH589), phenylbutyrate (PBA), OSU-HDAC42(AR-42)
HAT inhibitors: C646
HMT inhibitors: 3-deazaneplanocin A (DZNep)

of a single effector is insufficient to completely reverse the expression of specific miRNA. Therefore, many drugs that alter miRNA expression have been shown to work better in combination than when used individually (Wang et al. 2012). For instance, combination of trichostatin A (TSA) and 3-deazaneplanocin A (DZNep) induces the expression of miR-449 more dramatically than when these compounds are applied individually (Yang et al. 2009). Thus, the scheme of combining multiple epigenetic drugs to efficiently and effectively control altered miRNA expression in cancer cells appears to be attractive and should be investigated in detail.

Another strategy of controlling abnormal miRNA levels involves an oligonucleotide of 20nt antisense sequence with 2′-O-methyl modifications and phosphorothioate linkages. This molecule demonstrated effective inhibition of DNMT activity, reactivation of tumor suppressor functions, and inhibition of tumor growth during preclinical trials. The clinical trials for this therapeutic candidate was however unsuccessful due to its poor efficacy and high toxicity (Winquist et al. 2006). Improvement in the activity of this oligonucleotide along with reduction in associated toxicity is the subject of further studies. Additionally, drug candidates based on epi-miRNAs like miR-29s have also been developed (Amodio et al. 2015). These miRNAs are complementary to the 3′-UTR of DNMT mRNA and therefore block DNMT expression. These epi-RNAs, which are shown to have therapeutic potentials, are currently being tested for the treatment of hematological malignancies.

7.5 Conclusion

Unlike genetic lesions, epigenetic alterations in cancer can be reversed using molecules that inhibit specific epigenetic regulatory mechanisms. These candidate molecules have paved the way for novel therapies for cancer. The aim of such therapeutic candidates is to target the chromatin structure in a rapidly proliferating cancer cells with the intention of bringing them to a state similar to that of a noncancerous cell without causing any significant disturbance in the epigenome of healthy cells (Cortez and Jones 2008). The current research into epigenetics of cancer is continuously evolving our understanding and increasing our knowledge regarding the molecular basis of cancer, and epigenetic mechanisms have been elucidated as a key source for identification and development of newer therapeutic targets. The epigenetic proteins discussed in this chapter represent a number of potential targets for development of novel therapies. Furthermore, protein interactions that regulate different chromatin components can be novel targets for anti-cancer drugs.

In the cell, chromatin remodeling proteins often function as components of larger multiprotein complexes. Therefore, it is possible that inhibiting the activity of a single protein out of context would vary significantly from when that protein is present inside a multifunctional complex. In addition, these multiprotein complexes bind to specific locations on the chromatin in a tissue-specific manner. Depending upon the developmental and cellular context, the same chromatin-binding protein could function either as an oncogene or as a tumor suppressor. A complete knowledge of the biological roles of such target proteins and details regarding the mechanism of action of their inhibitor molecules still remains a challenge. Newer active chemical molecules with improved specificity are required which will be crucial in unveiling the biological functions of many novel proteins associated with chromatin and their implications in cancer development.

The epigenetic drugs so far developed that are approved or in advanced phases of clinical trials include inhibitors of DNMT, HDAC, HAT, HMT, and histone demethylase (HDT) enzymes (Boumber and Issa 2011). We have summarized a few epigenetic target-based drugs applied in cancer therapy in Table 7.3. Drugs based on inhibition of epigenetic modifications are already in clinical use as a combinatorial therapy along with established cancer chemotherapy. In addition, targeting the epigenome in some cases has shown direct reversal of resistance mediated by altered transcription. In spite of all the well-known limitations, present therapeutic based on targeting epigenetic mechanisms has demonstrated successful applications in cancer therapy. Further basic as well as translational research is required to discover newer avenues to fight against cancer.

Table 7.3 Epigenetic drugs used in cancer therapy

Tumor	Drug	Target	Clinical status	Reference
Breast cancer	Entinostat	HDAC1 and HDAC2	Phase II	Bradner et al. (2010)
	UNC0638	G9A (HMT)	Preclinical	Vedadi et al. (2011)
	DZNeP	EZH2 (HMT)	Preclinical	Fiskus et al. (2009) and Tan et al. (2007)
	dBET-1	BET (bromodomain)	Preclinical	Winter et al. (2015)
Colon cancer	Resminostat	HDAC1, HDAC3, and HDAC6	Phase II	Mandl-Weber et al. (2010)
	DZNeP	EZH2 (HMT)	Preclinical	Tan et al. (2007)
	GSK2801	BET (bromodomain)	Preclinical	SGC website
Lung cancer	Entinostat	HDAC1 and HDAC2	Phase II	Bradner et al. (2010)
	CG200745	Pan-HDAC	Phase I	Hwang et al. (2012)
	Mithramycin	SETDB1 (HMT)	Preclinical	Rodriguez-Paredes et al. (2014)
Prostate cancer	SB939	Pan-HDAC	Phase II	Wang et al. (2011)
	DZNeP	EZH2 (HMT)	Preclinical	Tan et al. (2007)
	GSK2801	BET (bromodomain)	Preclinical	SGC website
Ovarian cancer	GSK343	EZH2 (HMT)	Preclinical	Amatangelo et al. (2013)
	UNC669	L3MBTL1 (chromodomain)	Preclinical	Herold et al. (2011)
	GSK2801	BET (bromodomain)	Preclinical	SGC website
Neuroblastoma	BIX-01294	G9A (HMT)	Preclinical	Lu et al. (2013)
Pancreatic cancer	Belinostat	HDAC1, HDAC2, HDAC3, and HDAC6	Approved	FDA.gov
	BRD4770	G9A (HMT)	Preclinical	Yuan et al. (2012)

Lymphoma	Belinostat, panobinostat	HDAC1, HDAC2, HDAC3, and HDAC6	Approved	FDA.gov
	Romidepsin	HDAC1, HDAC2, HDAC3, and HDAC8	Approved	FDA.gov; Bradner et al. (2010)
	Mocetinostat	HDAC1 and HDAC2	Phase II	Bradner et al. (2010)
	EPZ005687, EI1	EZH2 (HMT)	Preclinical	Knutson et al. (2012)
	EPZ-6438	EZH2 (HMT)	Clinical trials	Knutson et al. (2012)
	UNC1215	L3MBTL3 (chromodomain)	Preclinical	James et al. (2013)
Leukemia	Belinostat	HDAC1, HDAC2, HDAC3, and HDAC6	Approved	FDA.gov
	Vidaza (5-azacytidine)	DNMT	Approved	Fenaux and Ades (2009)
	Decitabine (5-aza-2′-deoxycytidine)	DNMT	Approved	Shen et al. (2010)
	Guadecitabine, SGI-110	DNMT	Phase II	NCT01261312
	EPZ00477, SGC0946	DOT1L (HMT)	Preclinical	Daigle et al. (2011)
	EPZ-5676	DOT1L (HMT)	Clinical trials	Daigle et al. (2011)
	Chaetocin	SUV39H1 (HMT)	Preclinical	Chaib et al. (2012)
	JQ1, I-BET151, dBET-1	BET (bromodomain)	Preclinical	Filippakopoulos and Knapp (2012), Winter et al. (2015) and Chaidos et al. (2014)
	I-BET762	BET (bromodomain)	Clinical trials	NCT01943851NCT10587703
	Tranylcypromine	LSD1 (HDM)	Preclinical	Schenk et al. (2012)
	ORY-1001	LSD1 (HDM)	Clinical trials	Helin and Dhanak (2013)
Melanoma	DHAC (5-6-dihydro-5-azacytidine)	DNMT	Phase II	Creagan et al. (1993)
Liver	Resminostat	HDAC1, HDAC3, and HDAC6	Phase II	Mandl-Weber et al. (2010)
ALL	AR-42	HDAC (Class I and II)	Phase I	Lucas et al. (2010)

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Anticancer Immunotherapy: Prospects and Challenges

8

Ruchi Sachdeva, Poonam Singh, Anjali Bose, Beneeta Kalha, Moumita Sarkar, and Rahul Pal

Abstract

Accumulating evidence indicates that immune cells in the microenvironment play a significant role in tumor progression. Understanding the dynamic interplay between tumor cells and the host immune system is therefore of critical importance. While some immunotherapeutic strategies aim to specifically overcome hyporesponsiveness to tumor-associated antigens, others seek to nonspecifically activate the immune system; efficacy and toxicity are overriding, and often conflicting, concerns. This chapter, while occasionally providing brief historical perspectives, describes ongoing efforts in the area, including strategies for anticancer vaccination and adoptive cell transfer, the use of cytokines and development of oncolytic viruses, and the administration of checkpoint inhibitors. While significant successes have been achieved, the field is also characterized by notable failure, an indication that much remains unknown about factors that trigger and promote tumorigenesis, about current limitations in the effective delivery of therapeutic moieties, and about the workings of the immune system.

Keywords

Immunotherapy · Tumor-associated antigens · Vaccines · Chemotherapy · Adoptive cell therapy · Oncolytic viruses

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8.1 Introduction

Tumor cells arise through a combination of genetic and epigenetic changes that facilitate and drive immortality. Numerous oncogenes and tumor suppressor genes have been characterized in human cancers. For example, while the major isoforms of the Ras family of small GTPases are ubiquitously expressed in animal cells, mutated Ras proteins (observed in about 15% of human malignancies) are associated with a malignant phenotype (Disis and Cheever 1996). Tumor suppressor genes, on the other hand, work to decrease invasiveness and metastases (Osborne et al. 2004). For example, the tumor suppressor gene *P53* (also called transformation-related protein 53, TRP53) suppresses tumorigenesis in its wild-type configuration (Lane and Levine 2010), encoding a crucial transcription factor which exerts control over the process of apoptosis (Soussi et al. 2010); *P53* mutations are present in about 50% of human malignancies.

Mutation events in tumors can lead to the creation of neo-antigens which can render neoplastic cells detectable by the immune system. Although the immune system is capable of recognizing even subtle changes on the surface of transformed cells, such cells can escape immune destruction via multiple mechanisms, including immune evasion, the active induction of tolerance, as well as the disruption of T-cell signaling; as a consequence, the endogenous activation of tumor-specific immune cells can sometimes be suboptimal. Perhaps as a consequence of the appreciation of such active immune suppression mechanisms, anticancer intervention was initially restricted to the methods of surgery, radiation therapy, and chemotherapy. Emerging understanding of the causes of neoplastic transformation and of the workings of the immune system are leading to approaches that, in isolation or conjunction, seek to harness the power of the immune system for prophylactic or therapeutic benefit. A range of immunotherapeutic approaches have been employed over the years, including the administration of monoclonal antibodies and anticancer vaccination, the use of cell-based therapies, and oncolytic viruses. The therapeutic efficacies of nonspecific immune stimulatory agents (such as BCG, cytokines, and immune checkpoint inhibitors) have also been evaluated (Fig. 8.1).

8.2 Anticancer Vaccines

Many anticancer vaccines have been licensed for use, with many more being currently evaluated.

8.2.1 Vaccination Against Cancer-Inducing Infectious Agents

Cancers that arise as a consequence of viral infection present an ideal opportunity for prophylactic anticancer vaccination. Infection with human papillomavirus (HPV) and hepatitis B virus (HBV) has been associated with the development of cervical cancer and hepatocellular carcinoma, respectively. The US Food and Drug

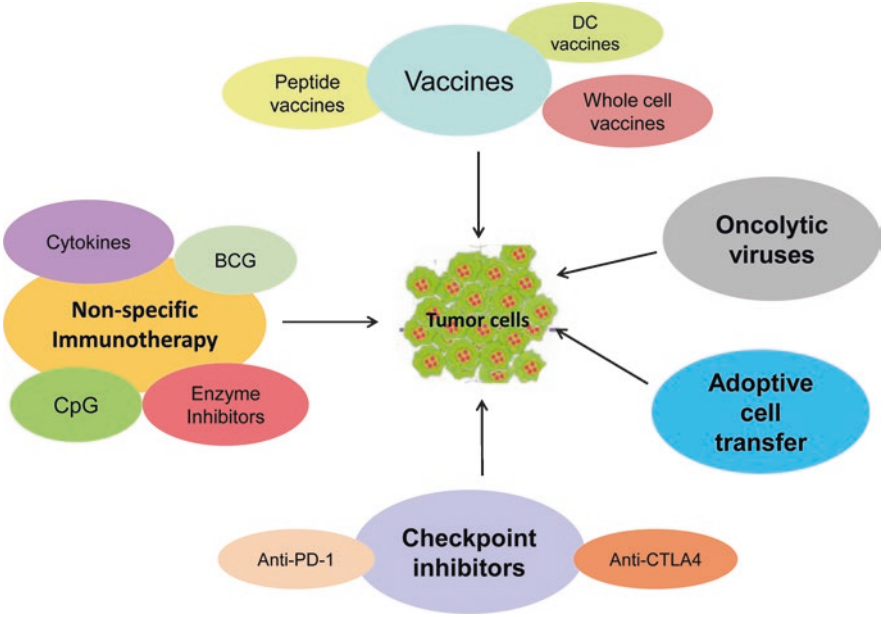

Fig. 8.1 Anticancer immunotherapeutic strategies. The range of approaches include anticancer vaccination, adoptive cell transfer, immune checkpoint inhibitors, oncolytic viruses, and cytokines

Administration has approved two prophylactic vaccines against HPV (Gardasil and Cervarix) which provide protection against HPV16 and HPV18; together, these viruses account for about 70% of cases of cervical cancer worldwide (Doorbar 2006; Parkin 2006). Both vaccines are composed of viral subunit-like particles, comprising the viral capsid protein L1. Immunologic responses were evaluated with a formulation consisting of quadrivalent HPV (types 6, 11, 16, 18) L1 virus-like particle (VLP) vaccine. Vaccine-induced anti-HPV responses were approximately 12- to 26-fold higher than those observed in naïve women (Villa et al. 2006).

Chronic infection with HBV is associated with 80% of all liver cancers (Frazer et al. 2007; Cutts and Hall 2004). The use of anti-HBV vaccination as an anticancer prophylactic measure began in 1981 and constitutes the earliest such intervention of its kind. The anti-HBV vaccine, based on 22-nm particles containing the recombinant HBV surface antigen (HBsAg), is highly immunogenic.

Epstein–Barr virus (EBV) is the primary cause of infectious mononucleosis and is associated with epithelial cell malignancies such as nasopharyngeal carcinoma and gastric carcinoma, as well as with lymphoid malignancies including Hodgkin lymphoma (Cohen 2015). In a study of patients with advanced nasopharyngeal cancer, an autologous monocyte-derived dendritic cell-based vaccine, consisting of HLA-A1101-, A2402-, or B40011-restricted epitope peptides from EBV latent membrane protein 2, was injected into the inguinal lymph nodes. Epitope-specific cytotoxicity was detected in patients, which coincided with a partial reduction in

tumor volume (Lin et al. 2002). More recently, the efficacy of EBV-stimulated cytotoxic T-lymphocyte immunotherapy was evaluated in patients. Though specificity for tumor-associated antigens was observed, the overall response rate for metastases in patients was low (Huang et al. 2017).

8.2.2 Vaccines Against Tumor-Associated Antigens

Since, in the majority of instances, no clear link exists between tumorigenesis and an infectious agent, the development of effective antitumor vaccines is a daunting challenge. By their very definition, such vaccines would have to be therapeutic (rather than prophylactic) in nature, requiring the generation of immune responses in a potentially compromised immune environment. In this regard, most strategies have tended to focus on the elicitation of T-cell immunity. It stands to reason that since peptides differentially bind different HLA alleles, ideal candidates for peptide vaccines would consist of HLA-"compatible" peptides that are derived from tumor-associated antigens (TAAs) expressed exclusively (or primarily) on tumor cells; additionally, such peptides should be able to induce cytotoxic T-cell responses upon immunization. This is easier said than done; tumor cells largely express self-antigens to which the immune system has previously been tolerized. Though the expression of TAAs may not be entirely restricted to tumor cells, mutations or relative abundance may make them suitable targets for immunological intervention. For example, mutations in oncogenes that drive the process of tumorigenesis, such as activating mutations in RAS, represent attractive targets for cancer immunotherapy. Immunization with recombinant yeast expressing mammalian-mutant Ras proteins causes complete regression of Ras mutation-bearing lung tumors in a dose-dependent manner (Lu et al. 2004).

A great deal of attention has been paid to developmental antigens (such as tyrosinase, melan-A, and gp100), cancer-testis antigens (such as MAGE, NY-ESO-1, and SPAG-9,) and oncofetal antigens (such as human chorionic gonadotropin, carcinoembryonic antigen, and α-fetoprotein) in this regard (Sobol 2005). MAGE-A3 is a cancer-testis antigen (Simpson et al. 2005) that is expressed in significant levels only in the testes, where it remains inaccessible to T cells because of a relative lack of MHC expression; immune tolerance to MAGE-A3 is therefore generally absent. MAGE-A3 is expressed in approximately 35% of lung tumors, and expression correlates with tumor stage. Peptide-based immunotherapy targeting MAGE-A3 has been shown to result in tumor regression (Marchand et al. 1995). However, MAGE-A3 immunotherapy does not increase disease-free survival compared with placebo in patients with MAGE-A3-positive surgically resected non-small cell lung cancer (Vansteenkiste et al. 2016).

The testis-specific sperm-associated antigen 9 (SPAG9), a member of the c-Jun NH(Osborne et al. 2004)-terminal kinase-interacting protein family, plays a functional role in sperm-egg fusion and in the mitogen-activated protein kinase signaling pathway. SPAG9 is up-modulated in cervical carcinoma (Garg et al. 2009), colorectal carcinoma (Kanojia et al. 2011), bladder transitional cell carcinoma

(Kanojia et al. 2013), and epithelial ovarian carcinoma (Garg et al. 2007). SPAG9 is also overexpressed in human prostate cancer and appears to contribute to the growth of prostate cancer cells (Li et al. 2014). More recently, its overexpression has been positively correlated with metastasis in hepatocellular carcinoma (Yan et al. 2016). Results of clinical trials in patients of cervical cancer are awaited.

In a recent Phase I clinical trial in patients of hepatocellular carcinoma, a vaccine consisting of glypican-3 (GPC3)-derived CTL epitopes was able to induce measurable immune and clinical responses; levels of IFN-γ were also found elevated. Moreover, the presence of the specific Th cell correlated with prolonged overall survival (Sayem et al. 2015).

Since antigen and/or HLA down-modulation can occur on tumor cells, simultaneous immunization with multiple peptide antigens has been attempted. A recent Phase II study carried on patients of metastatic renal cell carcinoma involved a pool of 9 HLA-A-02-restricted peptides and 1 HLA-DR (MHC Class II)-restricted peptide. When administered along with GM-CSF, the vaccine induced T-cell responses that positively correlated with tumor regression and survival (Walter et al. 2012).

8.2.3 Tumor Cell-Based Vaccines

Vaccination with irradiated, autologous tumor cells has been attempted. Potential limitations of this approach include difficulty in obtaining cells in large amounts and inability to reproducibly generate vaccine components free of contaminants. "Canvaxin," a melanoma vaccine comprising three irradiated, allogeneic melanoma cell lines, when administered along with BCG, induces both humoral and cell-mediated immune responses to the melanoma-associated tumor antigen TA90 (Hsueh et al. 1998); cytokines have sometimes been coadministered (Berinstein 2007). An irradiated, syngeneic, GM-CSF-expressing tumor cell vaccine (Gvax) has been shown to evoke a potent and sustained antitumor immune response in melanoma patients (Lipson et al. 2015). In a Phase II study of Belagenpumatucel-L, a *TGFβ-2* antisense gene-modified allogeneic tumor cell-based vaccine for non-small-cell lung cancer, survival of patients was enhanced as a result of TGFβ inhibition in the tumor microenvironment, resulting in DC activation and the suppression of Treg activity (Neumatis et al. 2006).

RNA, derived from renal cell tumors, was used to transfect autologous DCs. In Phase II trials with this vaccine ("AGS-003"), the incorporation of sunitinib (a multi-target tyrosine kinase inhibitor employed as conventional therapy for RCC) was shown to enhance patient survival (Figlin et al. 2012).

8.2.4 Vector-Based Vaccines

Most viruses are naturally immunogenic, and several have been shown to be capable of infecting dendritic cells. Infection with recombinant viruses engineered to express tumor antigens and/or cytokines can result in an increase in the frequency

and avidity of tumor-directed cytotoxic T lymphocytes. In Phase III trials, intratumoral vaccination of melanoma patients with T Vec ("talimogene laherparepvec," composed of herpes simplex virus encoding GM-CSF) resulted in systemic antitumor immune responses upon induction of tumor cell lysis (Andtbacka et al. 2013).

"TroVax" (a vaccinia virus expressing the 5T4 tumor-associated antigen), when injected in patients of metastatic renal cell carcinoma (RCC) and colorectal carcinoma, induced 5T4-specific antibody responses, which correlated with increased patient survival (Hawkins et al. 2009; Harrop et al. 2008). The utility of another recombinant vaccinia virus-based vaccine ("TG4010") expressing MUC-1 along with IL-2 has been assessed in RCC patients. This vaccine, when administered in combination with exogenous IFN-α and IL-2, generated higher MUC-1-specific T-cell responses and enhanced survival (Oudard et al. 2011; Ramlau et al. 2008), and diminished the progression of prostate cancer as demonstrated by an enhanced doubling time of serum PSA (Dreicer et al. 2009). TG4010 combined with chemotherapy (paclitaxel and carboplatin) improved survival (Quoix et al. 2016).

Immunization of patients of metastatic, castration-resistant prostate cancer with "TRICOM" (a recombinant vaccinia virus containing genes for prostate-specific antigen (PSA), B7.1, ICAM-1, and LFA-3) resulted in enhanced median overall survival (Kantoff et al. 2010). Preclinical studies have previously shown that "TRICOM" more efficiently generated antitumor T-cell responses and antibody responses as compared to the vaccines with one or no costimulatory molecules. "Prostvac" was evaluated in patients with prostate cancer. Patients were treated with an initial dose of "rV-PSA-TRICOM" (recombinant vaccinia) and then with "rF-PSA-TRICOM" (recombinant fowl pox); survival benefits were observed (Madan et al. 2009).

8.2.5 Dendritic Cell (DC)-Based Vaccines

Many cancer immunotherapeutic strategies have targeted DCs in attempts to induce antigen-specific immune responses. Preparation of DC vaccines can be achieved by "loading" tumor-associated antigens onto patients' autologous DCs which are then administered to patients. Antigens utilized for this purpose include tumor-derived proteins or peptides (Schuler-Thurner et al. 2002), whole tumor cell lysate (Salcedo et al. 2006), and DNA/RNA/viruses (Su et al. 2005). Tumor cell-DC fusions have also been employed (Rosenblatt et al. 2011).

One of the first trials assessing the immunogenicity of DCs was carried out in patients of metastatic prostate cancer. Some subjects injected with autologous DCs pulsed with HLA-A0201-restricted peptides derived from prostate-specific membrane antigen demonstrated antigen-specific cellular responses (Murphy et al. 1996). "Sipuleucel-T" was approved in 2010 for the treatment of asymptomatic metastatic castrate-resistant prostate cancer; an increase in survival was observed (Longo 2010). The efficacy of human Langerhans cells (LCs) as immunogens was compared with monocyte-derived dendritic cells (moDC) in melanoma patients; though both vaccines proved safe and immunogenic for melanoma antigens, LCs induced T-cell stimulation and IL-15 production, whereas moDCs required

exogenous IL-15 for clinical efficacy (Romano et al. 2011). Vaccination with DCs loaded with synthetic peptides for glioma-associated antigen (GAA) epitopes and polyinosinic-polycytidylic acid [poly(I:C)] stabilized by lysine and carboxymethylcellulose (poly-ICLC) induced positive immune responses against at least one of the vaccination-targeted GAAs in several patients (Okada et al. 2011).

The large number of peripheral blood monocytes-derived DCs required in such approaches frequently represents a significant roadblock. Genetically modified iPS-DCs may circumvent this limitation; cytotoxic T cells arising in carcinoembryonic antigen (CEA) transgenic mice immunized with mouse pluripotent stem cell-derived dendritic cells expressing CEA (miPSDCs-CEA) displayed antitumor activity against murine adenocarcinoma cells (Kitadani et al. 2018).

8.2.6 DNA-Based Vaccines

Bacterial DNA itself acts as a pathogen-associated molecular pattern, inducing the activation of immune cells (Barber 2011). DNA vaccines can be rationally combined with other immune-stimulatory agents (such as TLR agonists) to optimize immune responses.

A DNA cancer vaccine targeting HER-2/Neu, when used in conjunction with the TLR9 agonist IMO, resulted in control of HER2-positive mammary carcinoma in a murine model (Aurisicchio et al. 2009). Much of the same outcomes were observed in a murine model of CEA-positive colon carcinoma when a DNA vaccine targeting CEA was coadministered with the TLR7 agonist SM360320 (Dharmapuri et al. 2009).

DNA vaccines designed to target HER-2/Neu to costimulatory B7 molecules on APCs (by fusion with the extracellular domain of CTLA-4) induced amplified CD8 T-cell responses, providing protective immunity and delaying the onset of HER-2/Neu-driven mammary carcinoma (Sloots et al. 2008).

A DNA vaccine expressing vascular-endothelial growth factor receptor 2 (FLK-1) promoted the CTL-mediated killing of endothelial cells, resulting in therapeutic efficacy against several murine tumors (melanoma, colon carcinoma, lung carcinoma) (Niethammer et al. 2002). Oral administration of a xenogenic DNA vaccine encoding the human tumor endothelial marker TEM8 effectively suppressed tumor angiogenesis and protected mice from subsequent challenge with a lethal dose of tumor cells (Ruan et al. 2009).

Human telomerase reverse transcriptase (TERT) is overexpressed in more than 85% of human cancers regardless of cellular origin. Immunization with "INVAC-1," an optimized DNA plasmid encoding an inactivated form of TERT, reduced the growth of sarcomas and increased the survival rates (Thalmensi et al. 2016).

Although DNA vaccine strategies have shown some promise in several preclinical studies, they have generally failed to induce adequate therapeutic benefit in non-human primates and in humans (Rice et al. 2008). However, new constructs and methods of administration may enhance their utility; in addition to subcutaneous or intradermal injection, DNA vaccines can be injected directly into the lymph nodes to increase uptake by APCs and promote local inflammatory signals (Weber et al.

2011). Other modalities, including the use of a gene gun, electroporation, the use of ultrasound and lasers, as well as the employment of liposomes, microparticles, and nanoparticles, have been used to enhance the efficacy of DNA vaccination (Greenland and Letvin 2007).

8.2.7 RNA-Based Vaccines

Messenger RNA (mRNA) from autologous tumor tissues can be used to induce specific CTL responses (Wolff et al. 1990). RNA vaccination is usually carried out in conjunction with agents that enhance stability and/or mediate adjuvant effects, such as liposomes or protamines (Fotin-Mleczek et al. 2012). Integration of an RNA replicase polyprotein derived from the Semliki Forest virus to generate "self-replicating" RNA (Ying et al. 1999), or use of β-globin UTR to provide additional stability (Carralot et al. 2005), has also been shown to enhance antigen-specific immune responses. Protamine-stabilized mRNAs coding for melan-A, tyrosinase, gp100, MAGE-A1, MAGE-A3, and survivin were intradermally injected into melanoma patients; the frequency of Treg cells was significantly decreased, and an increase of vaccine-directed T cells was observed, accompanied by antitumor responses (Weide et al. 2009).

8.2.8 Antihormone Vaccines

After conception, human chorionic gonadotropin (hCG) extends the life of the corpus luteum, eliciting the secretion of progesterone (Braunstein 1996). It also plays important roles in implantation and angiogenesis during pregnancy (Berndt et al. 2006). Due to the critical functions it serves in pregnancy, hCG has been considered an ideal target for immunocontraception. Our lab has extensive experience in the design and evaluation of anti-hCG vaccines as antifertility agents (Singh et al. 1989; Talwar et al. 1997; Alam et al. 1989; Pal et al. 1990; Pal and Singh 2001). A vaccine comprising of *βhCG* associated non-covalently with the alpha subunit of ovine-luteinizing hormone (conjugated to tetanus toxoid or diphtheria toxoid as carriers) induced high-avidity, bioneutralizing antibodies against hCG. In fertile women, only one pregnancy was observed in 1224 recorded menstrual cycles when antibody titers were above cutoff; fertility was regained as titers fell (Talwar et al. 1994), and babies subsequently born exhibited normal developmental parameters (Singh et al. 1998), evidence that the anti-fertility effects were reversible.

Unexpectedly, hCG (or, more often, its individual subunits) has been found to be synthesized by several non-trophoblastic carcinomas (Cole 2012). Synthesis is seen in poorly differentiated and high-grade tumors and is associated with chemoresistance (Szturmowicz et al. 1999), radioresistance (Moutzouris et al. 1993), and poor prognosis (Douglas et al. 2014). Work in our lab has established that hCG can act as a growth factor for tumor cells in vitro and can up-modulate the transcription and expression of several pro-tumorigenic genes, including those implicated in

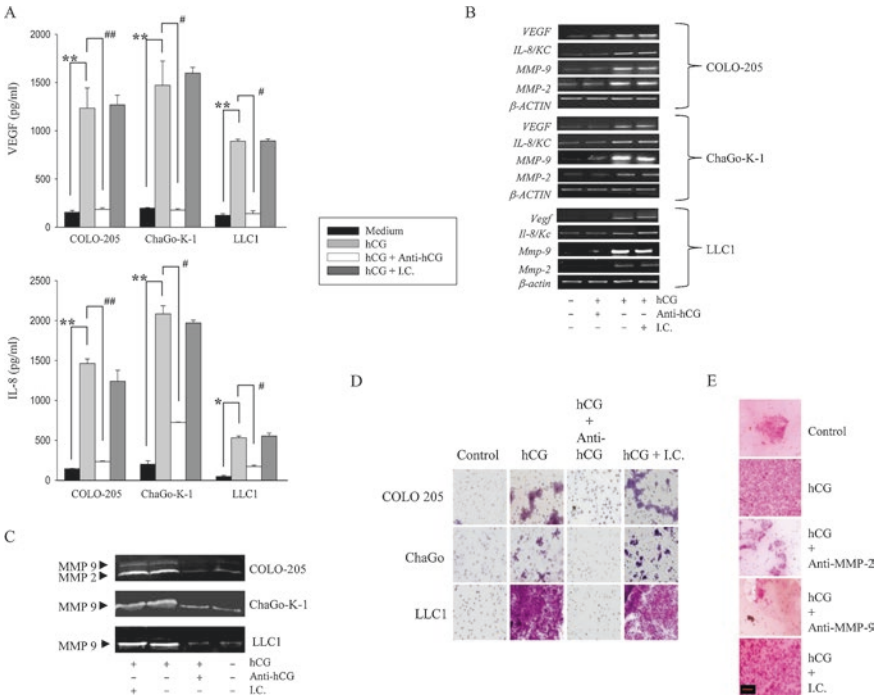

Fig. 8.2 (**a**) *VEGF* (top panel) and *IL-8/KC* (bottom panel) secreted from COLO-205 (human colorectal cancer), ChaGo-K-1 (human lung cancer), and LLC1 (murine lung cancer) cells upon incubation with hCG. The influences of anti-hCG antibodies and isotype control (I.C.) antibodies on hCG-induced effects are also shown. $^{*}p<0.05$, $^{**}p<0.001$, $^{\#}p<0.05$, $^{\#\#}p<0.01$. (**b**) mRNA transcripts (as assessed by reverse transcriptase-PCR) of *VEGF*, *IL-8/KC*, *MMP-2*, and *MMP-9* in COLO-205, ChaGo-K-1, and LLC1 cells upon incubation with hCG. The influences of anti-hCG antibodies and isotype control (I.C.) antibodies on hCG-induced effects are also shown. (**c**) *MMP-2* and *MMP-9* production (as assessed by zymogram analysis) from COLO-205, ChaGo-K-1, and LLC1 cells upon incubation with hCG. The influences of anti-hCG antibodies and isotype control (I.C.) antibodies on hCG-induced effects are also shown. (**d**) Invasion of COLO-205, ChaGo-K-1, and LLC1 cells into Matrigel upon incubation with hCG. The influences of anti-hCG antibodies and isotype control (I.C.) antibodies on hCG-induced effects are also shown. (E) The effects of anti-*MMP-2*, anti-*MMP-9*, and isotype control (I.C.) antibodies on hCG-induced invasion of COLO-205 cells into Matrigel. Bar = 50 μm. (Data from Khare et al. 2017)

chemoresistance; anti-hCG antibodies, as well as specific siRNAs, can neutralize such effects (Fig. 8.2) (Sahoo et al. 2015; Khare et al. 2017). *βhCG* demonstrates growth-factor-like properties on bladder cancer cells (Gillott et al. 1996), and anti-*αhCG* antibodies as well as *αhCG* antisense oligonucleotides have antitumorigenic effects on lung tumor cells (Rivera et al. 1989). While antibodies to *βhCG* inhibit the growth of *βhCG*-expressing tumor cell lines and prolong survival of tumor-bearing mice, active immunization against hCG reduces tumor growth (Acevedo et al. 1987). Choriocarcinoma cells transfected with *βhCG* antisense oligonucleotides undergo enhanced apoptosis, and anti-hCG antibodies can also induce the killing of uterine cervix adenocarcinoma cells (Kalantarov and Acevedo 1998). Vaccination with

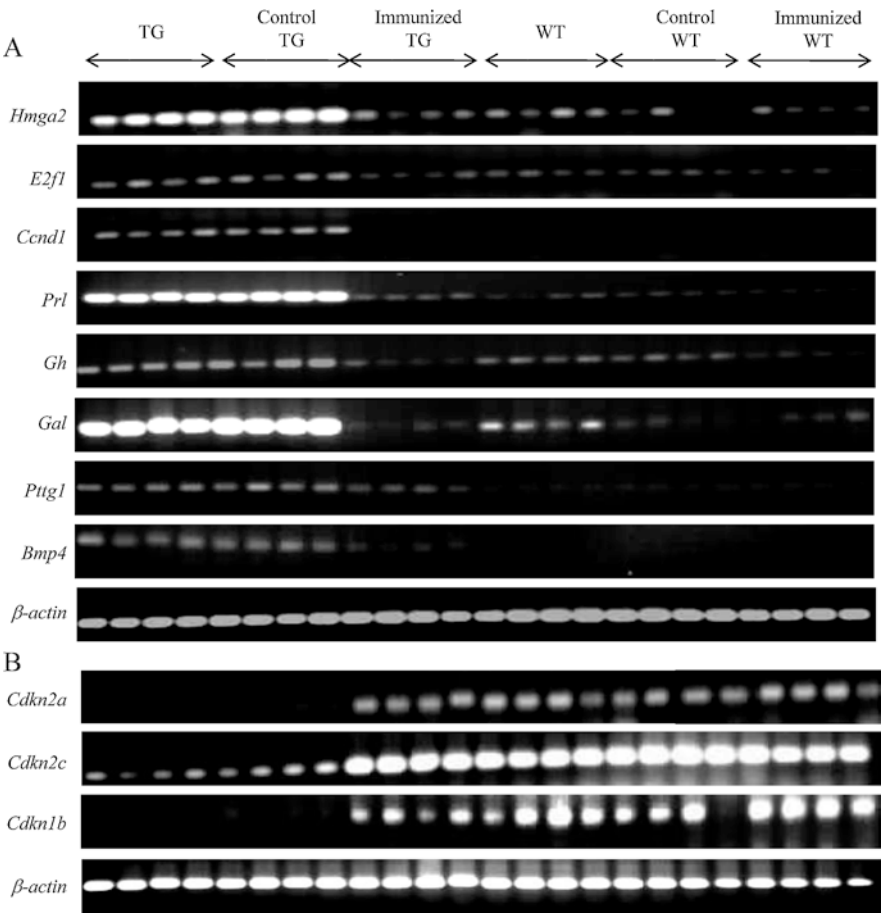

Fig. 8.3 Effects of active immunization against hCG on tumor-associated pituitary transcripts in *βhCG* transgenic (TG) and wild-type (WT) mice. (A) mRNA transcripts of tumor-associated proteins and (B) CDK inhibitors in pituitaries derived from TG mice, TG mice immunized with IFA ("Control TG"), TG mice immunized with hCG + IFA ("Immunized TG"), WT mice, WT mice immunized with IFA ("Control WT",) and WT immunized with hCG + IFA ("Immunized WT"). Each lane represents an individual animal. (Data from Sachdeva et al. 2012)

CTP37-DT (the carboxy-terminal peptide of hCG conjugated to diphtheria toxoid) in patients with advanced colorectal cancer induces anti-hCG antibodies and enhances survival (Moulton et al. 2002). Studies in our lab have described the preventive effects of anti-hCG immunization on the development of ovarian dysfunction and the onset of pituitary tumorigenesis in *βhCG* transgenic mice (Fig. 8.3) (Sachdeva et al. 2012). More recently, our lab has demonstrated the advantages of combining anti-hCG immunization with *Mycobacterium indicus pranii* (MIP) administration (Bose et al. 2013), and synergistic, antitumor effects of anti-hCG immunization + MIP combined with chemotherapy have been documented (Sahoo et al. 2015).

Gonadotropin-releasing hormone (GnRH)-based vaccines represent a promising therapeutic alternative for patients of prostate, ovarian, and breast cancers; B-cell tolerance toward the molecule can be broken by carrier conjugation. Immunization of rats with GnRHm1-TT (GnRH mutated at position 6, attached to amino acids 830–844 of tetanus toxoid) induced significant reduction in the growth of Dunning R3327 tumors (Junco et al. 2008). Similar effects were achieved upon immunization with GnRH linked to promiscuous T-cell epitopes derived from four natural pathogens (Finstad et al. 2004). Immunization of female rabbits with an immunogen containing the pseudomonas exotoxin A receptor-binding domain and 12 copies of GnRH resulted in the generation of high-titer antibodies against GnRH; such intervention may prove beneficial in the treatment of GnRH-sensitive ovarian cancers (Hsu et al. 2000). Active immunization of bonnet monkeys with GnRH, conjugated with diphtheria toxoid, induced high titers of anti-GnRH antibodies with subsequent reduction in testosterone levels as well as prostate size (Giri et al. 1991). Immunization of rats with a modified GnRH analog (D-lys at position 6) conjugated to DT via an epsilon-aminocaproic acid linker resulted in a decline in testosterone levels as well as a reduction in the size of the testes and of accessory sex organs (Jayashankar et al. 1989). Immunization of rats with a recombinant vaccine comprising of five units of GnRH (combined with T-helper cell peptides from the *Plasmodium falciparum* circumsporozoite protein, TT, respiratory syncytial virus, and measles virus) induced decreases in testosterone levels and prostatic atrophy (Talwar et al. 2004). In patients with advanced prostate cancer, GnRH-directed vaccination induced response-related reduction in testosterone levels (Simms et al. 2000) as well as a fall in prostate-specific antigen and clinical improvement (Talwar et al. 2009).

8.2.9 Combination of Chemotherapy and Vaccination

Evidence suggests that the immunomodulatory properties of some chemotherapeutic agents can be exploited to enhance the effects of anticancer vaccination. Such synergy can be brought about by multiple mechanisms and can depend on the type of cytotoxic agent and the vaccine employed, as well as on the dosing schedule of each agent. Combination of an anti-CEA vaccine with chemotherapy (cisplatin plus vinorelbine) resulted in the differential modulation of the hemostatic peripheral expansion of the T effector cell and Treg cell subsets; such synergy resulted in enhanced CEA-specific immune responses against lung carcinoma (Gameiro et al. 2011). Further, in a preclinical murine model of NSCLC, the combination of this chemotherapeutic doublet and vaccine increased survival of tumor-bearing mice, an effect mediated by both CD4 and CD8 T-cell subsets. The cisplatin plus vinorelbine combination has also been clinically evaluated in conjunction with a recombinant modified vaccinia Ankara vaccine expressing mucin-1 (MUC-1) and IL-2 (Gebbia et al. 2008).

Cyclophosphamide, doxorubicin, and paclitaxel, when used in combination along with a whole tumor cell vaccine, enhanced the antitumor immune response in a preclinical model of breast carcinoma (Machiels et al. 2001). Low-dose

cyclophosphamide and doxorubicin, administered alongside a GMCSF-secreting HER2/neu-expressing whole tumor cell vaccine in patients with metastatic breast cancer, demonstrated low toxicity; HER2-specific immunity was induced (Emens et al. 2009).

"Navitoclax," a Bcl-2 inhibitor, acts by mimicking the pro-apoptotic BH3 domain (Chonghaile and Letai 2008), targeting Bcl-2 and Bcl-2-related proteins such as Bcl-xL and Bcl-w (Oltersdorf et al. 2005; Reed and Pellecchia 2005). Its administration enhanced the efficacy of peptide-pulsed DC vaccination in a CT26 colon carcinoma model. "GX15-070" is a synthetic derivative of bacterial prodiginines (Fineran et al. 2007). It binds several Bcl-2 family members, including Bcl-2, Bcl-xL, Bcl-w, Mcl-1, and BAK (Nguyen et al. 2007), and induces apoptosis in hematologic and solid tumor cells in vitro and in vivo. "GX15-070" can mediate an increase in immune-mediated antitumor activity by decreasing Treg cell-dependent immune suppression. An increased intra-tumoral-activated CD8:Treg cell ratio was observed in mice first vaccinated with "rV/F-CEA/TRICOM" and then treated with "GX15-070" (Farsaci et al. 2010), effectively reducing pulmonary tumors in immune-competent mice.

An adenovirus-based DNA vaccine induced strong cell-mediated immune responses against dog telomerase reverse transcriptase; combination of vaccination with a cyclophosphamide/vincristine/prednisone regimen led to an increase in survival (compared with dogs receiving chemotherapy alone) of dogs affected by B-cell lymphosarcoma (Gavazza et al. 2013).

8.3 Adoptive Cell Therapy

Adoptive cell therapy (ACT) involves the isolation of T cells from patients, their genetic modification, and/or activation in vitro, followed by their reinfusion. In mice as well as in humans, ACT can, in ideal cases, result in the clearance of long-lived tumors.

8.3.1 Lymphocytes

In early experiments, treatment of rats bearing sarcomas with lymphocytes isolated from immunized syngeneic rats was shown to result in complete eradication of tumors (Delforme and Alexander 1964). C57BL/6 mice receiving a lethal dose of Friend virus-induced leukemia (FBL-3) cells survived upon transfer of autologous splenic T cells immune to FBL-3 (Berenson et al. 1975). Long-term culture of peripheral blood cells from melanoma patients in the presence of IL-2 and irradiated melanoma cells led to the generation of cytotoxic T cells capable of lysing autologous melanoma cells (Hérin et al. 1987). Treatment of relapsing chronic myeloid leukemia (CML) patients with IFN-α along with allogeneic PBMC resulted in long-term remission (Kolb et al. 1990). In patients of Hodgkin's disease after a first relapse, autologous bone marrow transplantation resulted in significant survival

benefit (Reece et al. 1994). In an attempt to increase the therapeutic response in myeloma patients, vaccination against tumor antigen was followed by adoptive transfer of bone marrow cells from allogeneic hosts, resulting in enhanced survival (Neelapu et al. 2005). Autologous T cells, co-stimulated using CD3 and CD28 antibodies, were infused in patients of multiple myeloma following chemotherapy and hematopoietic cell transplantation; lymphocytosis was delayed, and complete or partial responses were observed in twelve out of sixteen patients (Laport et al. 2003). Both CD4 and CD8 T cells have been employed for the purposes of ACT; though tumor-reactive Th1 and Th2 cells are capable of inducing reduction in tumor volume, it appears that both require the presence of cytotoxic CD8 T cells (Nishimura et al. 1999). Lymphodepletion, mediated upon the action of various drugs, is known to enhance the efficacy of transferred cells. In mouse myeloma model, such preconditioning serves to enhance levels of IL-7Rα on adoptively transferred CD4 T cells; exogenous IL-7 increases therapeutic effects (Ding et al. 2017). Transfer of Epstein–Barr virus (EBV)-cytotoxic T lymphocytes induced complete remission in 11 of 13 patients of EBV-positive lymphoproliferative disease; transferred cells persisted for nine years (Heslop et al. 2010). Therapeutic efficacy has also been reported in patients of EBV-expressing Hodgkin's lymphoma. Administration of EBV-specific T cells led to remission; evidence of an expansion of transferred cells in vivo was obtained, along with a reduction in viral load (Bollard et al. 2004). Interestingly, tumor antigen-specific naïve T cells mediated more potent effects than did central memory cells, attributed to enhanced proliferation, cytokine production, and reduced senescence (Hinrichs et al. 2009). CD19 is a frequent target of ACT strategies, and co-expression of cytokines like IL-7 and IL-21 can augment the effect of CD19-recognizing T cells (Markley and Sadelain 2010). Non-cytolytic CD4 T cells specific for P97 (a melanoma antigen) induced remission when administered to mice carrying pulmonary metastatic lesions, possibly with the assistance of antigen-presenting cell-derived cytokines (Kahn et al. 1991). T cells specific for minor histocompatibility antigens, administered to treat relapsing leukemia subsequent to hematopoietic stem cell transplant, induced complete (but transient) remission in most cases but also caused severe toxicity (Warren et al. 2010).

8.3.2 Lymphokine-Activated Killer (LAK) Cells

Lymphokine-activated killer cells constitute peripheral blood lymphocytes which are isolated and cultured in vitro in presence of IL-2 prior to reinfusion; IL-2 is frequently coadministered into patients as well. Natural killer (NK) cells are believed to comprise the main effector population of LAK cells. Autologous NK cells, when transferred into the patients of glioma, were shown to extend survival (Ishikawa et al. 2004). Some evidence suggests that survival benefits may increase upon transfer of haploidentical (as opposed to autologous) NK cells (Miller et al. 2005). LAK NK cells, when infused into patients of renal cell carcinoma previously treated with IL-2, induced antitumor responses and complete remission, though not in all cases (Escudier et al. 1994). Recurrence of hepatocellular carcinoma after

surgery is associated with significant mortality; transfer of autologous lymphocytes following in vitro expansion in presence of IL-2 resulted in a significant reduction in tumor recurrence (Takayama et al. 2000). Postsurgical administration of LAK cells (followed by chemo- or radiotherapy) was found to be of benefit in patients of lung cancer, enhancing survival rates over patients receiving standard therapy (Kimura and Yamaguchi 1997).

8.3.3 Tumor-Infiltrating Lymphocytes

Tumor-infiltrating lymphocytes (TILs) are populations of immune cells isolated from tumors, cultured in vitro in the presence of cytokines (such as IL-2), and then reinfused (Rosenberg et al. 1986).

Upon transfer of tumor-reactive TILs to patients of melanoma, longitudinal studies established the long-term survival of a $CD8^+$, $CD27^+$, and $CD28^+$ cell population, which probably mediated antitumor effects (Powell Jr. et al. 2005). TILs isolated from melanoma patients and grown in vitro in presence of recombinant IL-2 were also shown to mediate cytotoxic effects on allogeneic tumor cells. Such TILs could be expanded up to 95,000-fold and still retained the ability to induce tumor lysis in an HLA-restricted manner; cytolytic activity was restricted to melanoma cells, with peripheral blood cells remaining unaffected (Muul et al. 1987). Some studies suggest that, upon transfer, telomere length and the expression of CD28 are associated with TIL persistence and antitumor responses (Shen et al. 2007).

Lymphodepletion, frequently employed prior to TIL infusion, has been shown to enhance antitumor effects (June 2007); cyclophosphamide and fludarabine (with or without total body irradiation) have been employed in patients of melanoma. In 20 of 93 patients with complete regression, 5-year survival rates were close to 100% (Rosenberg et al. 2011).

8.3.4 Recombinant T Lymphocytes

Jurkat T cells, when transfected with the alpha and beta T-cell receptor chains derived from a melanoma-reactive T-cell clone specific for MART-1 (a melanoma-specific antigen), were shown capable of recognizing MART-1 peptides (Cole et al. 1995). Cloning of MART-1-specific TCRs in primary T cells or TILs induced lysis of tumors in an HLA-restricted manner (Hughes et al. 2005). Retroviral transfection of a TCR specific for gp100 (another melanoma-specific antigen) in primary human lymphocytes induced similar results; transfection of TILs afforded on them a supplemental anti-gp100 reactivity (Morgan et al. 2003). Targeting the cancer-testis antigen NY-ESO-1 in a similar manner generated cells that could lyse a wide spectrum of NY-ESO-1-expressing tumor cells (Zhao et al. 2005). In another study, engineered anti-NY-ESO-1-specific CD4 T cells, when introduced into patients of metastatic melanoma, induced tumor regression, even though only 50%–75% of the

tumor cells were shown to express the antigen; analysis revealed that, upon the infusion of anti-NY-ESO-1-specific CD4 T cells, T cell responses against other melanoma antigens such as MART-1 and MART-3 were initiated due to as yet unexplained mechanisms (Hunder et al. 2008).

An interesting strategy seeking to circumvent the PD-1-PD-L1 suppressor axis (believed to be responsible for both the failure of endogenous antitumor T-cell responses and the failure of adoptive T-cell therapies) has been proposed. Transgenic T cells specific for tumor-expressed antigen were engineered to express a PD-1-CD28 construct; treatment of mice-bearing subcutaneous tumors resulted in complete regression (Kobold et al. 2015). Recognition of the suppressive effects of TGFβ has led to protocols which involve coadministration of tumor-targeting transgenic T cells and the TGF-beta receptor-I kinase blocker SM16; significantly greater in vivo expansion of transferred cells was observed, accompanied by a reduction in tumor volume (Wallace et al. 2008).

As with other strategies, lymphodepletion enhances the efficacy of adoptively transferred transgenic tumor-reactive T cells as well. The reasons for such effects are unclear; while endogenous cells possibly act as "sinks" for critical cytokines such as IL-7 and IL-15 (Gattinoni et al. 2005), there is also evidence to suggest that the effects of lymphodepletion on gut microflora plays a role (Paulos et al. 2007).

8.3.5 Chimeric Antigen Receptor (CAR) T Cells

An scFv fragment of a monoclonal antibody, when expressed on cytotoxic T lymphocytes along with appropriate cytoplasmic sequences capable of triggering T-cell-specific activation signals, constitutes a chimeric antigen receptor; the great advantage of such a strategy is recognition (for example, of a tumor cell surface antigen) is not MHC-restricted. In brief, tandem variable region fragments of light and heavy chains of a tumor-reactive antibody are cloned in conjunction with a cytoplasmic signaling domain (Eshhar et al. 1993). Anti-HER-2 CAR T cells were generated employing an scFv fragment derived from monoclonal antibody to anti-HER/Neu along with the transducing subunits of either the CD3 ζ chain or the Fc receptor γ chain. Coculture of these CAR T cells with tumor cells expressing the antigen induced the production of IL-2 by the former and lysis of the latter (Stancovski et al. 1993). Anti-CD19 CAR T cells have been employed with some success in refractory chronic lymphocytic leukemia and B-cell acute lymphoblastic leukemia (Grupp et al. 2013); response rates as high as 88% have been achieved with some anti-CD19 CAR T cell approaches. Inclusion of the costimulatory domain CD137 along with the CD3 ζ chain may have beneficial effects; when employed in anti-CD19 CAR T cells, such a strategy resulted in a 1000-fold cellular expansion of these cells in patients of chronic lymphocytic leukemia, with cells persisting over 6 months; in two of three patients, decreased plasma cell numbers were accompanied by complete remission (Kalos et al. 2011). Though the expression of CD19 is infrequent in multiple myeloma, some reports do suggest expression on drug-resistant cells; remarkably, administration of anti-CD19 CAR T cells (along with chemotherapy and autologous stem cell transplantation) to

a patient with refractory multiple myeloma resulted in antitumor effects, despite the absence of CD19 on 99.95% of tumor cells (Garfall et al. 2015). Integration of the extracellular ligand-binding domain along with domains with the CD3 ζ chain, CD28, CD4, and lck resulted in increased receptor sensitivity and potency (Geiger et al. 2001). In particular, the inclusion of the CD28 domain was shown to enhance both expansion and persistence of CAR T cells upon infusion (Haynes et al. 2002; Savoldo et al. 2011). The adding of a 4-1BB domain helps dampen T-cell exhaustion (Long et al. 2015). Inclusion of OX40 along with the CD3 ζ chain and CD28 domains appears to provide further benefit (Pulè et al. 2005). An interesting variation on the strategy is the introduction of antitumor chimeric receptors into EBV-specific CTLs; such dual receptor-bearing cells demonstrate enhanced efficacy when administered to patients of neuroblastoma, most likely due to continuous stimulation by EBV-infected cells in vivo (Pule et al. 2008).

Several factors influence the efficacy of CAR T cells. In non-myeloablative therapy regimens, nonresponders tend to express a higher proportion of Treg cells; irradiation acts to reduce these levels and enhance efficacy (Dotti et al. 2014).

Solid tumors pose several additional challenges for such therapies, including the presence of myeloid-derived suppressor cells and inhibitory cytokines, a hypoxic environment, as well as extracellular components which can negatively influence CAR T-cell function (Burga et al. 2015). Additionally, tumors frequently down-modulate targeted antigens; for example, HER2-null cells emerge upon the targeting of the molecule on glioblastoma cells. Dual targeting strategies have been devised in attempts to circumvent this phenomenon; HER2- and IL-13Rα2-specific CAR T cells (administered as a pool of two different CAR T cells or with constructs co-expressed in the same CAR T cell) demonstrate enhanced efficacy against autologous glioblastoma cells in vitro as well as in murine models (Hegde et al. 2013). A similar strategy has been attempted in the dual targeting of Erb2 and MUC1 on breast cancer (Wilkie et al. 2012).

The aberrant growth of blood vessels is believed to contribute greatly to tumor expansion, serving to provide vital growth-promoting signals. Prostate-specific membrane antigen (PSMA) is expressed on prostate cancer cells, as well as on endothelial cells in many solid tumors, and absent on normal vasculature; CAR T cells targeting PSMA eliminate tumor cells by direct action and also act on tumor vasculature to reduce tumor burden (Santoro et al. 2015). Pancreatic cancer cells express prostate stem cell antigen (PSCA) at the early stages of malignant transformation. Though CAR T cells targeting PSCA in human pancreatic tumor-bearing immunodeficient mice effectively cause tumor lysis, concerns about off-target effects have been expressed (Abate-Daga et al. 2014). Local delivery of cells may also help in some instances; mesothelin-targeted CAR T cells were much more efficacious when administered intrapleurally as opposed to intravenously in a preclinical lung cancer model (Adusumilli et al. 2014). Co-expression of the CCR2 receptor in CAR T cells targeting neuroblastoma enables such cells to home into CCL2-producing tumor cells following which tumor lysis occurs with higher efficiency (Craddock et al. 2010). Supplementing CAR T-cell therapy directed against neuroblastoma with an oncolytic virus expressing RANTES and IL-15 improves

outcomes in a preclinical model; the virus exerts direct tumor cytopathic effects, and intra-tumoral cytokine release serves to attract CAR T cells and also prolongs their survival (Nishio et al. 2014).

Several strategies have been devised to deal with side effects encountered upon CAR T-cell therapy. Serological markers such as C-reactive protein may help in management of cytokine-release syndrome (CRS), symptoms of which include high fever, cardiac dysfunction, and renal failure (Davila et al. 2014). Severe cases of CRS are effectively treated with anti-IL6 antibodies or the IL-6R antagonist tocilizumab (Lee et al. 2014). Injecting free antibodies targeting the antigen before the transfer of CAR T cell may help in reducing liver toxicity (Lamers et al. 2013). Neurological toxicity, including confusion, delirium, and seizure, has been reported upon transfer of anti-CD19 CAR T cells. These effects are generally self-resolving and may be caused by heightened cytokine levels (Bonifant et al. 2016). In a combinatorial targeting approach to reduce toxicity, T cells were transduced with two CARs, one each for PSMA and PSCA. While the presence of a single antigen was suboptimal for activation, the presence of both antigens triggered a killing response; such dual-molecule sensing serves to enhance tumor specificity (Kloss et al. 2013). Prolonged persistence of transfused CAR T cells is occasionally associated with graft versus host disease (GVHD), a condition with potentially grave consequences. Inclusion into CAR T cells of a construct comprising caspase 9 fused to FK506-binding protein renders them susceptible to apoptosis by a synthetic dimerizer ligand (Di Stasi et al. 2011). Methods of ameliorating off-target effects without elimination of transferred cells have also been devised and become important when tumor-associated antigens are also expressed on normal tissue. Inhibitory CAR (iCAR) constructs contain CTLA-4 or PD-1 cytoplasmic domains attached to extracellular domains, recognizing antigens present on normal tissue and down-modulated on tumor tissue; HLA class I has been considered in this regard, as has OPCML-v1, which is present on normal tissue but demonstrates curtailed expression in lymphomas, breast cancer, and prostate cancer. Co-expression of CARs and iCARs on T cells can serve to preserve efficacy while reducing toxicity (Fedorov et al. 2013).

Besides those outlined above, CAR T cells have been employed to target several other antigens (for example, CD20, CD22, CD30, ROR1, CD123, CD33, CD133, CD138, the κ light chain, the B-cell maturation antigen) present on a variety of cell types, with varying degrees of success.

8.4 Oncolytic Viruses

Interestingly, signaling cascades that promote the process of tumorigenesis overlap with those that serve to enhance viral replication. Oncolytic virus therapy seeks to use replicating viruses to selectively target cancer cells without causing harm to normal tissue (Russell et al. 2012). While anticancer strategies increasingly seek to combine oncolytic virus therapy with tumor antigen vaccination, immune checkpoint inhibitor therapy, or adoptive cell therapy (Lichty et al. 2014), this review focuses more on the basic principles behind the concept of oncolytic viruses.

Actively replicating viruses such as HSV-1, vascular stomatitis virus (VSV), poxviruses, adenoviruses, measles virus, poliovirus, Newcastle disease virus, and vaccinia virus can be reprogrammed to mediate antitumor effects using three approaches: "targeting," "arming," and "shielding" (Kaufman et al. 2015).

"Targeting" involves modifying a viral component or choosing a virus subtype which has a natural tropism towards a particular cancer; such an action is aimed at improving both safety and efficacy. For example, many tumor lineages overexpress CD46, which serves as a receptor for the measles virus (Anderson et al. 2004). The Edmonston vaccine strain of measles virus, when administered to patients with drug-refractory, recurrent ovarian cancer, induced a decrease in CA-125 levels and extended patient survival (Galanis et al. 2010). Negating natural viral tropism is frequently required, however (Vongpunsawad et al. 2004), and the acquisition of cancer cell tropism can be mediated by an appropriate scFv. For example, measles virus with an incorporated anti-CD20 scFv was effective in a human xenograft tumor model (Bucheit et al. 2003). A measles virus carrying an scFv toward the EGF receptor has also been developed (Nakamura et al. 2005). Inclusion of viral glycoproteins gD and gB in oncolytic herpes simplex virus leads to efficient targeting of glioblastoma multiforme tumor cells via the epidermal growth factor receptor (Uchida et al. 2013).

Another strategy to enhance specificity utilizes the fact that viruses require proteolytic cleavage of cell-targeting ligands before attachment and entry, a function that ubiquitous cell-derived proteases normally provide. Introduction of matrix mellatoproteases (MMP)-cleavable sites in the measles virus envelop fusion protein enhances tumor targeting (Springfeld et al. 2006); since MMPs are overexpressed in many cancers, such methodologies can be broadly applicable. Urokinase-type plasminogen activator, another enzyme expressed at high levels by many cancer lineages (Andreasen et al. 2000), can also be exploited in this regard. Introducing transcriptional control of virus replication using preferentially expressing cancer-specific (or, in some cases, tissue-specific) promoters has also been assessed as a strategy. For example, the expression of the adenoviral E4 under the telomerase promoter results in selective replication of the virus in tumor cells (Kuppuswamy et al. 2005). Adenoviral E1, expressed under the prostate-specific antigen promoter, results in viruses capable of specifically replicating in cells of prostatic lineage.

"Arming," as the name implies, seeks to incorporate prodrugs and/or cytokines into oncolytic viruses. Incorporation of the thymidine kinase gene into tumor-targeted viruses makes infected cells susceptible to the prodrug ganciclovir (Boviatsis et al. 1994). Introduction of pro-apoptotic genes (such as TRAIL) into oncolytic viruses can enhance potency both in vitro and in vivo (Ren et al. 2006).

Clinical trials suggest that the immune system plays a significant role in outcomes upon oncolytic virus therapy; the ability to induce immunogenic tumor cell death appears to be a correlate of efficacy. Incorporation of cytokines can help activate the immune system and aid in the reduction of tumor burden (Zhang et al. 1996). A herpes simplex virus producing GMCSF, designed to specifically replicate in tumor cells, enhanced survival when administered to patients of nonsurgically resectable melanoma (Andtbacka et al. 2015).

Newcastle disease virus has oncolytic properties and is a potent inducer of type I IFN. In a murine model of B16 melanoma, intra-tumoral injections of the virus resulted in leukocyte infiltration and antitumor effects even at distal sites; combination with systemic CTLA-4 blockade enhanced these effects and protected mice from rechallenge (Zamarin et al. 2014). In a similar murine model, dual CTLA-4 and PD-L1 blockade along with administration of an oncolytic measles virus also yielded added benefit (Engeland et al. 2014).

"Shielding" may involve treatment with immunosuppressive drugs prior to the infusion of oncolytic viruses to dampen preexisting antiviral immune responses or those arising as a consequence of therapy (Stanford et al. 2006). Other methods have also been adopted in efforts aimed at circumventing arising antiviral immune responses that may compromise efficacy; for example, sequential immunization with an adenovirus and an oncolytic VSV both expressing the same tumor antigen acts to boost immune responses to the tumor antigen at the expense of antibodies against VSV (Bridle et al. 2010).

Changing the viral serotype may also help circumvent preexisting antiviral immune responses, particularly as a consequence of multiple injections (Parks et al. 1999). Irradiated tumor cells infected with oncolytic viruses can act as carriers, preventing viral sequestration and inactivation by immune components in the bloodstream. Additionally, such cells can preferentially home to the tumor site (Munguia et al. 2008).

8.5 Nonspecific Immunotherapy for Cancer

Nonspecific immunotherapies aim to broadly activate the immune system to achieve anticancer effects; they can involve both the innate and adaptive arms of the immune system. Some such strategies are discussed in the following sections.

8.5.1 Immune Checkpoint Inhibitor Therapy

The discovery of immune checkpoints is considered a major advance in the field of immunobiology. During T-cell activation, immune checkpoint molecules transduce inhibitory signals, counteracting stimulatory signals. CTLA-4 (a molecule homologous to CD28, also known as CD152) and PD-1 (also known as CD279) are most representative of the immune checkpoint mediators on T cells (Du et al. 2018). In the tumor microenvironment, ligands for these molecules (CD80/86 and PD-L1, respectively) can be present in abundance and work to skew the immune response, preventing effective antitumor immunity (Adachi and Tamada 2015). CTLA-4 competes very efficiently with CD28 for binding to CD80/CD86 on antigen-presenting cells (Kavecansky and Pavlick 2017). The binding of CTLA-4 to CD80/CD86 impedes the co-stimulation signal provided by CD28-CD80/CD86 interaction, resulting in T-cell anergy (Beyersdorf et al. 2015). CTLA-4-CD80/86 interaction disrupts IL-2 production and cell cycle progression (Krummel and Allison 1996).

PD-1 ligand (PD-L1, also known as CD274) binds PD-1 to initiate a signaling cascade that prevents the phosphorylation of key intermediates in the T-cell signaling pathway, ultimately promoting apoptosis (Buchbinder and Desai 2016). Overexpression of PD-L1 on the tumor cells can suppress antitumor immune responses (Patel and Kurzrock 2015). PD-1-PD-L1 interactions are believed to play an important role in tumor progression and survival (Alsaab et al. 2017).

"Ipilimumab" is an anti-CTLA-4 antibody and the first such drug to be approved for clinical use (Hodi et al. 2010; Robert et al. 2011); its administration has been shown to improve survival in patients with metastatic melanoma (Hodi et al. 2010). Other studies have revealed evidence of continued disease stabilization or regression 5–10 years after initiation of therapy (Schadendorf et al. 2015).

More than 500 clinical studies have assessed the effects of the disruption of PD-1–PD1-L1 interaction on about 20 distinct tumor lineages (Callahan et al. 2016). Antibodies against PD-1 have been shown to mediate significant antitumor effects in RCC, melanoma, NSCLC, and colorectal cancer (Gettinger et al. 2014; Topalian et al. 2014). In advanced melanoma, anti-PD-1 antibodies induced superior clinical responses than anti-CTLA-4 antibodies; response rates were 33–34% in patients treated with "pembrolizumab" (an anti-PD-1 antibody), higher than response rates (12%) in patients treated with "ipilimumab" (Farolfi et al. 2012; Ribas et al. 2016). Such antibodies have also demonstrated efficacy in non-Hodgkin's lymphoma, multiple myeloma, and acute myeloid leukemia (Berger et al. 2008; Westin et al. 2014). Patients with unresectable melanoma receiving "nivolumab" (another anti-PD-1 antibody) demonstrated a 73% survival rate as compared to a 42% survival rate in the patients that received chemotherapy with dacarbazine (Robert et al. 2015). In patients with NSCLC, treatment with "atezolizumab" (an anti-PD-L1 antibody) resulted in significantly better survival rates compared to docetaxel (Hida et al. 2018).

Trials have been carried out to evaluate the efficacy of dual immune checkpoint blockade (anti-PD-1+ anti-CTLA-4) in patients who respond poorly to single blockade. In clinical trials conducted on patients with advanced melanoma, concurrent therapy with "nivolumab" and "ipilimumab" resulted in rapid and durable responses; about 53% of patients displayed greater than 80% reduction in tumor volume (Wolchok et al. 2013). In another Phase I study, combination therapy with "nivolumab" and "ipilimumab" in advanced melanoma patients demonstrated a 58% survival rate over a span of three years (Wolchok et al. 2017). Combination therapy with "nivolumab" and "ipilimumab" has also been approved for unresectable metastatic melanoma and is being assessed in patients of RCC (Hammers et al. 2014), NSCLC (Patnaik et al. 2015), and ovarian cancer (Iwai et al. 2017). In patients with metastatic renal cell cancer, "nivolumab" plus "ipilimumab" therapy demonstrated promising responses in terms of survival rate and safety; the objective response rate was 40.4%, and the two-year survival rate varied between 67.3% and 69.6% (Hammers et al. 2014). In Phase I clinical trials conducted on 78 NSCLC patients, "nivolumab" plus "ipilimumab" therapy resulted in high response rates (Hellmann et al. 2017).

8.5.2 Cytokines

8.5.2.1 Interferons

Type I IFNs (IFN-α, IFN-β) have emerged as the most clinically relevant interferons for the treatment of cancer. Type I IFNs induce expression of major histocompatibility complex (MHC) class I molecules on tumor cells and mediate the maturation of DCs (Trepiakas et al. 2009). Type I IFNs also exert apoptotic and antiangiogenic effects on the tumor neovasculature (Wagner et al. 2004). IFN-α induces apoptosis of tumor cells in a caspase-dependent manner in some instances while enhancing tumor cell antigen expression in others (Chawla-Sarkar et al. 2003). In addition to melanoma, IFN-α is approved for the treatment of some hematologic malignancies, AIDS-related Kaposi's sarcoma, and advanced renal cancer, the latter in combination with anti-*VEGF* therapy. IFN-α has been particularly effective in patients of hairy cell leukemia and chronic myelogenous leukemia (Quesada 1986). In some preclinical melanoma models, IFN-β was shown to be more potent than IFN-α in inducing antiproliferative effects (Chawla-Sarkar et al. 2001). IFN-γ is cytotoxic to some malignant cells and also has antiangiogenic activity (Friesel et al. 1987).

8.5.2.2 Interleukin-2

High-dose IL-2 induces objective clinical responses in 15–20% and complete responses in 5–7% in patients with advanced melanoma (Atkins et al. 2004). In melanoma and RCC patients, while the number of Treg cells increased after exposure to IL-2 and remained elevated in patients with disease progression, patients who responded to IL-2 demonstrated a decrease in Treg cells to normal levels within four weeks of treatment (Ahmadzadeh and Rosenberg 2006). High doses of IL-2, when given after anti-PD-1 therapy to melanoma and RCC patients with progressive disease, proved safe and effective (Buchbinder et al. 2016).

8.5.2.3 Interleukin-7

A potential therapeutic advantage of IL-7 over IL-2 is that it selectively expands CD8 T cells over Treg cells (Rosenberg et al. 2006). In murine cancer models, recombinant IL-7 has been found to augment antigen-specific T-cell responses after vaccination and adoptive cell therapy (Colombetti et al. 2009). Clinical trials on patients with advanced malignancy have demonstrated recombinant IL-7 to be well tolerated (Sportès et al. 2010). Co-expression of IL-21 and IL-7 in whole-cell cancer vaccines was shown to boost antitumor immunity in a $CD4^+$ and $CD8^+$ T-cell-dependent manner and generated effective memory in a murine model (Gu et al. 2016).

8.5.2.4 Interleukin-12

IL-12 has demonstrated antitumor activity in murine models of melanoma, colon carcinoma, mammary carcinoma, and sarcoma (Li et al. 2005a). IL-12 also exhibits antiangiogenic effects which are mediated by IFNs, particularly IFN-γ, and IP-10 (Kawamura et al. 1998). The cytokine acts to activate DCs, NK cells, and cytotoxic T cells (Alkayyal et al. 2016). Administration of a tumor-targeted oncolytic adenovirus (Ad-TD) expressing non-secreted IL-12 significantly enhanced the survival of

animals with pancreatic cancer (Wang et al. 2017). Combination therapy of IL-12 with immune checkpoint inhibitors and adoptive T-cell transfer may hold promise (Berraondo et al. 2018).

8.5.2.5 Interleukin-15

IL-15 acts to block IL-2-induced apoptosis in T cells (Marks-Konczalik et al. 2000) and supports the persistence of memory CD8 T cells (Ku et al. 2000). The cytokine has demonstrated significant therapeutic activity in several preclinical murine models of cancer (Di Carlo et al. 2000). Phase I trials with IL-15 have been initiated in patients with hematologic malignancies who relapse after allogeneic hematopoietic cell transplantation (Romee et al. 2018). In the presence of Treg cells, IL-15 (but not IL-2) promotes the proliferation and CTL effector function (Perna et al. 2013).

8.5.2.6 Interleukin-21

IL-21 has demonstrated therapeutic activity in murine tumor models of melanoma (He et al. 2006) and has been evaluated in clinical trials in RCC patients; significant antitumor activity was observed (Bhatia et al. 2014). IL-21 can play a role in adoptive T-cell strategies based on its ability to enhance the generation of T cells (Li et al. 2005b). Enhanced antitumor activity was observed when recombinant human IL-21 was combined with "cetuximab" (an anti-EGF receptor monoclonal antibody) in patients with stage IV colorectal cancer (Steele et al. 2012).

8.5.2.7 Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)

The expression of GM-CSF has been shown to provide protection to subsequent B16 tumor challenge in mice (Dranoff et al. 1993). Significant antitumor effects have been observed when GM-CSF has been employed in combination with other immunomodulators, such as checkpoint-blocking antibodies (Small et al. 1999). While "Gvax" (a GM-CSF-transduced tumor cell vaccine) independently primed tumor-reactive T effector cell function and delayed the growth of B16 melanoma, combination with CTLA-4 blockade induced greater immune cell infiltration to change the intra-tumor balance of Treg cells and T effector cells, an effect which directly correlated with tumor rejection (Quezada et al. 2006).

8.5.3 Mycobacteria

The use of mycobacteria in the treatment of cancer has been studied extensively; the basis of the efficacy of such intervention remains unclear, however. *Bacillus Calmette-Guérin* (BCG) is a live attenuated strain of *Mycobacterium bovis* widely used as a prophylactic vaccine against diseases induced by *Mycobacterium tuberculosis* (*M. tb*). In addition to stimulating specific antimycobacterial immunity, BCG is also believed to nonspecifically activate the immune system. It enhances the infiltration of granulocytes, macrophages, and T helper cells (Ratliff et al. 1993) while increasing the production of a wide spectrum of cytokines (Fuge et al. 2015).

BCG is employed in the treatment of superficial, non-muscle invasive bladder cancer (Ang et al. 2016). The current line of treatment for the high-risk bladder cancer is transurethral resection followed by intravesical BCG instillation (Bilsen et al. 2018); indeed, BCG instillation is considered the gold standard for the treatment of bladder cancer since it reduces both the progression and recurrence of disease (Shelley et al. 2010). Superior activity of BCG over transurethral resection alone or over transurethral resection and chemotherapy has been reported (Babjuk et al. 2017).

Intralesional administration of BCG is considered to be an effective therapy for the treatment of cutaneous metastatic melanoma (Triozzi et al. 2011). BCG instillation leads to inflammation and ulceration, followed by tumor regression (Lardone et al. 2017; Da Gama et al. 2018).

B16 melanoma cells, when transfected with a plasmid encoding the *M. tb* antigen early secretory antigenic target-6 (ESAT-6), secreted exosomes bearing both TAAs and ESAT-6. Such exosomes, when injected into footpads of mice, induced cellular immunity against both ESAT-6 and B16 tumor cells. Intra-tumoral injection of the exosomes significantly suppressed the growth of B16 tumors in comparison with exosomes derived from non-transfected tumor cells (Koyama et al. 2016).

8.5.4 CpG

CpG motifs constitute a pathogen-associated molecular pattern (PAMP); recognition of CpG by TLR-9 (Bauer and Wagner 2002) leads to the secretion of pro-inflammatory cytokines as well as the upregulation of maturation markers on antigen-presenting cells (Wagner 2001). CpG motifs are also present in the mammalian genome, although these are believed to be mostly methylated.

CpGs have been categorized into five different classes (Class A, Class B, Class C, Class P, and Class S) on the basis of sequences and secondary and tertiary structures (Vollmer and Krieg 2009). Cytokines arising as a consequence of CpG-TLR-9 interaction work to skew the immune response toward Th1 phenotype and promote CD8 T-cell responses (Adamus and Kortylewski 2018). Class A and Class B CpGs have been shown to enhance the efficacy of vaccination in patients with melanoma and those with lung, ovarian, breast, and colon cancers (Adamus and Kortylewski 2018; Scheiermann and Klinman 2014). CpG 7909, when administered as an adjuvant along with a melanoma antigen A (MELAN-A) analog peptide, led to stronger CD8 T-cell responses in melanoma patients (Speiser et al. 2005). Similar observations were made in patients of esophageal cancer; CpG, when administered along with recombinant NY-ESO-1 and montanide, led to enhancement in the tumor-specific antibody responses and NY-ESO-1-specific CD8 T-cell responses (Valmori et al. 2007); a similar trial reported an increase in life expectancy (Karbach et al. 2010). A lack of significant effects has also been reported, however. In clinical trials conducted on metastatic melanoma patients, no clinical benefit was observed when CpG-7909 was administered along with dacarbazine therapy (Weber et al. 2009), and only a slight enhancement in patient survival was observed when CpG was administered to patients of non-small cell lung cancer and metastatic colorectal cancer (Smith et al. 2014; Chan et al. 2015).

8.6 Conclusions

Immunotherapeutic strategies for cancer continue to evolve as investigators shed further light on the interactions of tumor cells with the immune system. Since TAA-targeted interventions are (in several instances) self-directed, autoreactivity is a potential concern. Nonspecific immune activation strategies too have a lot to offer, provided unrestrained inflammatory responses can be curtailed. This exciting area of research, at the cutting edge of both the basic and applied medical sciences, is at the crossroads, and next decade should help answer a critical question: Why do some patients demonstrate robust clinical responses to immunological intervention while others do not?

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Preclinical Animal Models for Cancer Research and Drug Discovery

9

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Abstract

Cancer has a complex biology and has been characterized by several hallmarks during its multistep progression such as sustaining proliferation, evading growth suppressors, resisting cell death, stimulating angiogenesis, activation of invasion and metastasis, etc. There is an increased understanding of complex biology of cancer in the past few decades; still this disease remains the primary cause of death in developed and developing countries all over the globe.

The efforts to reduce global cancer burden are mainly focused on developing innovative diagnostic and therapeutic tools. In this process of translation, animal models play a crucial role in translating diagnostic and therapeutic innovations to human clinical trials. Animal models are historically used in medical research initially for understanding of basic anatomy and physiology till developing new treatments for various diseases including cancer. The early twentieth century has seen a dramatic increase in the use of animal models, and since then the refinements of these models are happening untiringly. The refinements or improvements in model systems are required with increase in our understanding of human and animal biology as well as disease processes. This chapter deals with anticancer drug developments before and after arrival of immunocompromised animals in cancer research and various thought processes going on during the last century with respect to anticancer drug research. The authors have touched

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upon various advantages and disadvantages of model systems used in drug development. This chapter also includes current approaches towards cancer model systems along with upcoming models showing potential to expedite drug development process with reduction in attrition rate.

Keywords

Severe combined immunodeficient (SCID) · Non-obese diabetic (NOD) · Xenograft · Orthotopic · Patient-derived xenograft (PDX) · Transgenic model · Knockout mouse model · Canine spontaneous cancer

Cancer is a multistep development and has been characterized by several hallmarks: strong proliferative signalling, sidestepping growth suppressors, allowing replicative immortality, opposing cell death, tumour-promoting inflammation, stimulation of angiogenesis, activation of invasive and metastatic behaviour, genomic instability and mutations, avoidance of immune destruction and deregulation of cellular dynamics. Cancer is typically detected after structural differences in a tissue or organ using various diagnostic tools (Hanahan and Weinberg 2011).

Despite an improved knowledge of cancer with respect to its molecular aspects, immune environments and advanced therapies such as chemotherapy and immunotherapy, cancer remains the second major cause of mortality in the United States. As per the American Cancer Society, there were above 13.2 million deaths due to cancer in 2016, with a rate of 1800 people every day and close to 1,965,540 new patients, and it is expected to rise to 20 million cases by 2025.

There is increase in 5-year relative survival rate for all commonly diagnosed cancers during 2003–2009 from 49% to 68%. The 5-year survival rate of 49% was observed during 1975–1977. The key for this increase in patient survival rate is early cancer detection and precise treatment response monitoring (American Cancer Society Cancer 2014).

The efforts to reduce global cancer burden are mainly focused on developing innovative diagnostic tools. During this process of translation, animal models play a pivotal role in linking theragnostics innovations to human clinical trials. These animal models give sound understanding of varied aspects of the basic and clinical translational studies such as tumour biology, drug sensitivity, immunology, metastatic pattern and treatment response. These animal models are historically used on the basis of current knowledge of anatomy, physiology, biochemistry, genetics and immunology at that point of time. So along this journey, the animal model system has evolved to meet the requirement of modern medical research including cancer (Schachtschneider et al. 2017).

Identification of tumour-specific molecular targets is required to enhance early detection and designing anticancer-specific therapies for initial stages of the disease. This desires appropriate selection and use of in vitro and in vivo models in cancer research (Hanahan and Weinberg 2011). The initial use of animals started for the purpose of understanding the anatomy and physiology, which gave birth to

Table 9.1 Early developments and milestones in animal modelling (Schachtschneider et al. 2017; Ericsson et al. 2013; Krogh 1929; Storrs et al. 1974; Kikutani and Makino 1992; Orban et al. 1992; Doetschman and Azhar 2012; Ikawa et al. 1995; Saito et al. 2001; Filipiak and Saunders 2006; Wongsrikeao et al. 2011; Hong et al. 2009; Hammer et al. 1985; Wolf et al. 2000; Higashijima et al. 2000; Sasaki et al. 2009)

Period	Medical theorists/ researcher(s)	Study details
6th C BC	Alcmaeon of Croton	Studies using dogs to associate the brain with intelligence and sensory integration
4th C BC	Aristotle	Studied chick's embryogenesis and ontogeny
3rd C BC	Erasistratus	Worked on the cardiovascular system in live animals and explained functioning of the heart
2nd C AD	Galen of Pergamum	Extensively worked on cardiovascular and neuroanatomy using live animals
12th C	Avenzoar	Rehearsed surgical skills on animals prior human applications, e.g. tracheotomy
17th C	William Harvey	Extensively worked on comparative anatomy of different species of animals and established detailed functions of the cardiovascular and other systems
1902–1909	William Castle and Clarence Little	Initiated mice breeding for genetic experiments
1909	Frederick Banting	Started inbreeding of mice to eliminate experimental variation
1930	Little and MacDowell	Developed the first inbred mouse strain by brother-sister mating for 20 consecutive generations
1940s	John Cade	Investigated the anticonvulsant effect of lithium salts in guinea pigs. The knowledge gained was used to design treatments for depression
1976	Rudolf Jaenisch et al.	Development of transgenic mouse
1985	Daniel et al.	Extensive drug safety and efficacy studies conducted in rhesus macaques for HIV
1987	Capecchi, Evans and Smithies	Developed the first knockout mouse
1997	Wilmut and Campbell	First mammal cloning from an adult somatic cell of sheep
2002	Wade and Lagos-Quintana	Sequencing of mouse genome
2004	Blanchette	Sequencing of rat genome
2009	Aron Geurts et al.	Development of the first knockout rat

comparative medicine branch having foundation on the concept that animals and humans do share anatomical, physiological, behavioural, genetic and other characteristics. Table 9.1 provides the stepwise learning of molecular mechanism of diseases using animals around us.

The study of vertebrate mammals began in ancient Greece to understand human anatomy and physiology. These were mainly observation-based studies for understanding and comparing them with their human counterparts. The observation notes were well documented by prominent thinkers like Aristotle and could reach Europe

and other continents via trade routes, making animal models an indispensable research tool for like-minded European and Arabic physicians. As a consequence, this early period saw great medical discoveries (Ericsson et al. 2013; Krogh 1929).

The twentieth century began with a dramatic increase in the usage of animal models, which has also initiated a debate on their appropriateness as disease models and on ethics of their use in research. Unlike early days, when outbred animals were mostly used in research, the concept of inbreeding of animals started shaping up. Researchers such as William Castle, Clarence Little, Halsey Bagg and Leonell Strong could address the issue of genetic variability by developing inbred mice for research applications (Ericsson et al. 2013; Krogh 1929; Orban et al. 1992; Doetschman and Azhar 2012).

Abbie Lathrop, a self-made scientist, from Massachusetts, William Castle from Harvard University and Leo Loeb from the University of Pennsylvania started studying phenotypic traits on the basis of genetic determinants of inheritance in 1900. Clarence Cook Little developed inbred mouse strain for the purpose of his own tumour transplant study to reduce experimental alteration (Lunardi et al. 2014). The Jackson Laboratory was established by Little in the year 1929, which is now a place for advanced research to study genetics and genomics and has a mouse genome database. Scientists could decipher that there is 99% homology of mouse protein-coding genes with humans, consequently recognizing the importance of mouse as an animal model to study genetics and understand biology of various human diseases (Lunardi et al. 2014).

The initial anticancer treatments evolved from three ways of thought process. First was Ehrlich's 'magic bullet', to envisage target-specific drug development. This concept was designed on the basis of observations that certain dyes have varying affinities for different cellular components. This single observation generated opinion that some chemicals might selectively target neoplastic cells avidly than non-malignant cells (Decker and Sausville 2011).

It was observed that World War I soldiers who were exposed to mustard gas had severe leucopenia, which suggested effectiveness of these compounds against haematologic malignancies. A study in mouse models of lymphoid tumour showed promising regression of transplanted tumours when treated with nitrogen mustard gas. This observation stimulated introduction of alkylating agents in cancer treatment. Later in 1943 these agents were used in treatment of patients with non-Hodgkin lymphoma (Lunardi et al. 2014).

The third approach in developing anticancer treatment was on the basis of findings that hormonal manipulation regresses endocrine tumours in human patients. Later with the advent of molecular technologies, it was observed that the RNA content of malignant cells was several times higher than that of non-malignant cells. This resulted in identifying several new compounds with antimetabolic activity, which were further evaluated for anticancer drug efficacy. But during that time, the focus of pharma sector was primarily on non-oncology drugs (Decker and Sausville 2011).

Cancer drug development process suffered from two major roadblocks; the first one is scarcity of translational relevant models and the insufficient clinical trial infrastructure (DeVita and Chu 2008).

The various in vitro screening systems used for detection of active antitumour compounds such as cell or bacterial cultures were found inefficient, and it was also observed that no single model has predictable outcome for screening anticancer activity (Plowman et al. 1997). This has generated a need to design standard protocols for screening potential chemicals, which further resulted in establishing panels of tumour models. The use of serially passaged cancer cells in inbred murine hosts was envisaged for rapid screening studies. During initial phase, these screening methods were primarily used in academic institutes. After increased requirement for high-throughput screening of new compounds, the National Cancer Institute, USA, in 1955 initiated accessible resource about preclinical testing data and clinical trial details of new anticancer compounds and therapeutic strategies, which gave birth to the *Cancer Chemotherapy National Service Centre* and later renamed as *NCI Developmental Therapeutics Program*. Under this programme test compounds from various sources such as academic and pharmaceutical laboratories were evaluated, and potential compounds were further evaluated for the purpose of drug discovery (Decker and Sausville 2011).

Murine tumour screening systems acted as a mainstay during 1955–1975 (Plowman et al. 1997). Mouse origin cell lines such as L1210 and P388 were obtained from leukaemias generated in DBA/2 mouse by dermal topical application of methylcholanthrene. This procedure was quickly standardized for reproducible outcome for developing tumour-bearing animals by intraperitoneal inoculation of leukaemia cells (Liu and Hicklin 2011). The important clinically pertinent principles of anticancer chemotherapy were observed while working with mouse models and subsequently used as a basis of development of treatment protocols for human beings. The murine leukaemia model (P388) was extensively used as a primary screening tool till 1985 (Decker and Sausville 2011).

The drawbacks of mouse models started surfacing when molecules showing promising anticancer activity in mouse models started failing in clinical trials in humans conducted in the United States and other countries (Staquet et al. 1983). Similarly high attrition rate was observed by European cancer research organizations while conducting clinical trials (Winograd et al. 1987). Similarly in murine tumour models along with anticancer activities, the bone marrow and nontarget toxicities were observed with alkylating compounds and DNA-interacting molecules (Liu and Hicklin 2011).

The new observations such as poor efficacy of drugs in syngeneic mouse models but good treatment outcome in human cancer patients were seen with taxane compounds. The similar trend was found with respect to other compounds (Brefeldin and DNA minor groove binders). These observations started questioning the utility of murine models for development of anticancer drugs for human beings (Decker and Sausville 2011). Because of these observations, NCI conducted in-depth analysis of various tumour screening assays performed during 1976–1982. This analysis concluded about inadequacy of murine system in simulation of human malignancies

and in vivo complexities. Murine models were found to be not reliable in identifying antitumour activity as well as toxicities of the potential compounds (Plowman et al. 1997). There were several drawbacks found in mouse models such as rapid tumour growth, limited availability of tumour type and species-specific differences in drug metabolism and kinetics. On the basis of these observations, major changes have been made in anticancer drug screening system such as introduction of human tumour xenografts and solid tumour murine models along with leukaemia cell lines (Liu and Hicklin 2011).

The discovery of nude mouse potentiated the process of induction of xenotransplantation of human cancer cell lines and subsequently impacted global anticancer drug development programme (Rygaard and Poulsen 1969). The new screening panel consisting of solid transplantable tumours of human origin in immunocompromised nude mice was designed at NCI during 1976. The battery of screening tests developed further consisting L1210 leukaemia and B16 melanoma models along with matched animal and human tumours, namely, the breast, colon and lung. Besides, new protocol with implantation of human tumour fragment under renal capsule in mouse was initiated at NCI as a fast platform for new drug evaluation (Plowman et al. 1997).

The review of preclinical and clinical anticancer efficacy studies carried out during the mid-1980s pointed out differences in mouse and human studies conducted for potential therapies targeted to some tumour types. The reason for such discrepancies was thought to be limited types of tumour models used in the screening panel, which has improved the panel further by addition of more tumour types (Suggitt and Bibby 2005).

These learning lesions shifted the focus of European and Japanese groups in drug development towards disease-oriented use of human tumour xenografts from drug-oriented approach. During the early 1980s, researchers found a significant association between patient xenograft mouse model and individual patient response with respect to the drug efficacy (Fiebig 1988; Inoue et al. 1983). To tackle intricacies of anticancer drug development, in 1988 there was a formation of multicentre research collaboration in European countries consisting of Germany, the Netherlands, Norway, Scotland and Belgium. Under these collaborative efforts, studies were conducted for screening new molecules to assess preclinical drug response utilizing human tumour cell line xenograft panel. It was stated with caution that understanding of mechanism of drug action and discrepancies between the mouse model and human patient responses needs to be considered in designing appropriate tumour panel for drug screening (Boven et al. 1992). Simultaneously NCI also initiated a project on use of human cancer cell line panels for high-throughput anticancer drug screening. Combined efforts were made to strengthen NCI tumour repository by addition of subcutaneous murine xenografts from human tumour cell lines to the screening panels, and also cell lines of common cancer types such as leukaemia; brain, colon, lung, ovarian and renal cancer; and melanoma were added to a panel of in vitro and in vivo characterization testing, including drug sensitivity assays for the prospective screening (Plowman et al. 1997).

The usefulness of subcutaneous xenograft model increased due to the ease of experimental procedure while developing the model and also the practicability of manipulation in the cancer cells at genetic level prior to its transplantation. Besides, the availability of highly immunocompromised animals such as severe combined immunodeficient (SCID) and nonobese diabetic (NOD) SCID mice at affordable cost for cancer researchers proved to be an important aspect in cancer drug discovery research. The methodologies were further refined and improved for high-throughput screening of drugs in the 1990s.

NCI focused its strategy towards disease rather than a compound during the 1990s (Suggitt and Bibby 2005). They modified their existing in vivo anticancer drug screening assay consisting of 60 cell lines commonly referred to as NCI-60 and added few more prostate and breast tumour lines (Liu and Hicklin 2011). The data outcome of NCI-60 in vitro assay created a characteristic signature of drug responses with the help of a computer programme: COMPARE. This analysis demonstrates that compounds with similar signatures are also having similarities in chemical structures as well as mechanisms of action. On the basis of these findings, the association between drug efficacy and molecular target interaction was highlighted, which was further used to shift the drug discovery focus towards identification of molecular targets and characterizing tumour models on molecular basis so that relevant targets can be validated with candidate agents (Suggitt and Bibby 2005).

There was development of new assay systems for drug screening such as hollow fibre assay. In this assay multiple cell lines were used during in vivo pharmacodynamics and pharmacokinetic analysis. Basically in hollow fibre system, 12 cell lines were used and then implanted in murine system subcutaneously and intraperitoneally. The test compounds were dosed later and tumour tissue was harvested and analysed. The use of this assay system was initiated by NCI in 1995 for in vivo prescreening, and then on the basis of outcome of this assay, active compounds were then further taken for xenograft studies (Suggitt and Bibby 2005). Even after this refinement and comprehensive use of multiple cell lines, more than 85% of the screened drugs did not show activity. Hence to curtail on resource and time, prescreening system consisting of three highly sensitive systems was designed and introduced in practice during 1999 (Liu and Hicklin 2011). The validation of this approach was conducted by the National Cancer Institute of Canada Clinical Trials Group (NCIC CTG). The predictive value of commonly used screening systems such as in vitro cell lines and preclinical model (murine allografts and human tumour xenografts) was studied by NCIC CTG. The study revealed that in vitro cell line panel and the human xenograft models are predictive of phase II performance than the murine allograft models. This group recommended the use of the in vitro cell line complemented by incorporating relevant human xenograft models (Voskoglou-Nomikos et al. 2003). These days the standard drug screening of NCI consists of primary screening assay system as NCI-60 screening system, and after showing presence of activity in this assay, the next step is hollow fibre assay and then its subsequent antitumour activity in relevant human tumour xenograft mouse models in immunodeficient animals or other appropriate rodent model (NCI Dtp Drt

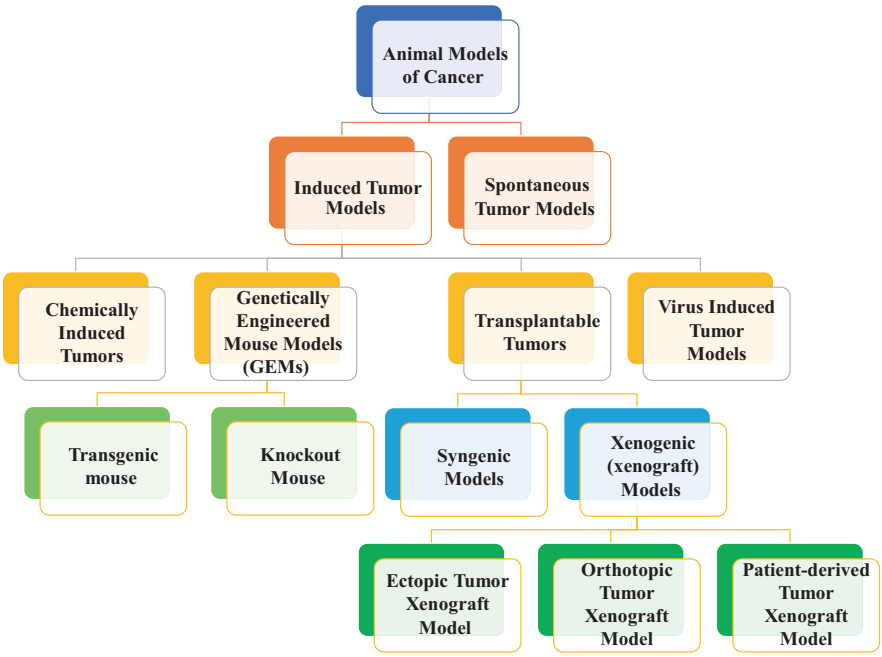

Fig. 9.1 Pictorial representation of commonly used animal models for cancer studies

2015). Most drug development programmes use the same basic strategy globally, with slight modifications, such as the Japanese Foundation for Cancer Research that utilizes similar panel of NCI-60 cell lines with addition of stomach and breast cancer cell lines representing high occurrence of disease in Japanese population (Kong and Yamori 2012).

The use of orthotopic xenograft models in rodents was introduced in subsequent evaluation of potential novel molecules after its initial standard assay protocol. At the NCI, the compound undergoes several steps of panel assays and also is assessed for available information/data for its anticancer activity evaluation and specific model conditions such as tumour types, drug administration route, dose and dosing frequency, etc. Generally, xenograft studies were initiated after identification target as well as mechanism of action (NCI Dtp Drt 2015).

Pictorial representation of commonly used animal models is shown in Fig. 9.1, and the details of important models are discussed further in this section below.

9.1 Chemically Induced Tumours

Animal models of chemical carcinogenesis are currently used for two major applications.

Firstly, they are used for experimental and translational oncology research; the main objective of this is identification of causes as well as mechanisms of cancer initiation to develop strategies for chemoprevention or early detection. Secondly, animal models are used to perform carcinogen bioassay for testing carcinogenic potential of the common compounds humans are getting exposed to.

The first study stating the link between environmental carcinogen exposure and cancer was published by Percivall Pott, an English physician and surgeon, in 1775. He noted that employees working as a chimney sweeper in their youth have long-term exposure to high concentrations of soot and tar, which led to skin cancer in scrotal region. His observation about latency indicated about multistage nature of cancer occurrence (Pott 1776).

Similarly in 1895 almost 100 years later, high incidence of bladder cancers was reported by Rehn in workers exposed to aniline dyes (Rehn 1895). Epidemiological data of these findings and other similar epidemiologic observations gave emergence to chemical carcinogenesis (Lawley 1994). Later the first paper was published stating direct link between chemical exposure and cancer in 1918 (Yamagiwa and Ichikawa 1918).

Yamagiwa and Ichikawa in their studies reported benign lesion and malignant epidermal tumours after long-term topical exposure of coal tar to rabbit ears. Similar studies performed in mice in the 1930s revealed polycyclic aromatic hydrocarbons (PAHs) benzo[a]pyrene, dibenz[a,h]anthracene and 3-methylcholanthrene as pure chemical compounds can lead to tumour formation in mice, establishing direct link between chemical compounds and a cause of cancer (Tsutsui 1918; Cook et al. 1932; Kennaway 1955).

Polycyclic aromatic hydrocarbons are having multiple complex benzene rings. They are produced during incomplete combustion process of organic matter. Air particulate matter and smoke from cigarettes have abundant quantity of PAH; they are widely dispersed in the environment and also in foodstuff. Due to widespread distribution of PAHs, few epidemiologic studies have linked their exposure and incidence of cancer in humans and establishment of their cancer-causing potential in experimental animal models. There were reports that there is occurrence of urinary bladder cancer in individuals working in the dye factories; on the basis of this observation, Sasaki and Yoshida in 1936 successfully designed experiments in rats and showed induction of liver cancer after feeding azo dye o-aminoazotoluene (Miller and Miller 1947). Later in 1941 Kinosita demonstrated induction of liver cancer after exposure to 4-dimethyl-aminoazobenzene (Kinosita 1936) and also reported in rats exposed to 2-acetylaminofluorine development of cancers of the bladder and other organ systems (Weisburger and Weisburger 1958). Studies of azo dyes demonstrated necessity of metabolic activation of cancer-causing entity in the host in vivo environment (Miller and Miller 1947).

Evidence of these animal studies about the role of aromatic amines in industry-related bladder cancer greatly reduced occupational exposure of industry workers to these cancer-causing compounds. The use of aromatic amines in chemical factories

and their presence in cigarette smoke are still greater concerns due to established evidence of cigarette smoking and prevalence of bladder cancer attributing to aromatic amines (Vineis and Pirastu 1997).

Furthermore, experimental studies carried out in animals on effect of different chemical exposures showed evidence of cancer after exposure to these environmental carcinogens. The use of sedative ethyl carbamate (urethane) was common during 1950–1975 in Japan, but experimental studies with mice exposed to urethane reported induction of lung cancers, which stopped the use of this chemical in human patients (Miller 1991). Urethane is present in low concentrations in many fermented foods. After its activation by the p450 system, vinyl carbamate epoxide binds covalently to DNA to form mutagenic adducts (Forkert 2010).

The other important class of chemical carcinogen is N-nitroso compounds. The first compound reported to be carcinogenic in rat models was N-nitrosodimethylamine (DMN) (Magee and Barnes 1956).

Many other compounds in this class were reported to induce cancer in almost all various species of animals used for testing (Magee and Barnes 1967). Nitrosamines produced during digestion and present in the environment may contribute broadly to cancer. Several animal studies in past such as X-ray-induced lymphomas, UV radiation-induced skin cancer, ovarian cancer and other types in mouse models pointed association of cancer and environmental exposures (Carr 1985; Findlay 1928).

Studies performed in the early to mid-twentieth century demonstrated absolute aetiological links between cancer development and exposure to chemical or radiation. These findings impacted on several aspects such as lifestyle changes and changes in regulations and industry practices for bringing down the cancer risk to humans (Homburger et al. 1962). The tumours induced in mouse using chemical carcinogens develop from the host's own cells; hence this model simulates human clinical scenario more appropriately than transplantable tumours. The important limitations to this method are possible effects of carcinogen upon the tumour biology and occupational hazards to personnel and also to other animals through direct exposure or excreted carcinogen and its metabolites in faeces and urine of the animals (Vollmer 2003).

Chemical carcinogens can be further categorized into direct-acting agents which do not require chemical transformation to induce carcinogenicity and indirect-acting agents which are active only after metabolic conversion.

There are agents such as procarcinogens having active end products known as ultimate carcinogens. These agents (direct-acting and ultimate carcinogens) are highly reactive electrophiles reacting with DNA, RNA and cellular proteins. Besides, these chemicals target genes such as RAS and TP53 genes, e.g. DMBA-induced mammary tumours in rats, DMAB-induced colon tumours, 3,4,9,10-dibenzopyrene-induced fibrosarcoma in mice, 3,4-benzopyrene-induced spindle cell sarcomas, 20-methylcholanthrene-induced leukaemia and sarcomas (Connors and Roe 1964; Nicol et al. 2004).

Mammary Tumours Induced Using DMBA (9,10-dimethyl-1,2-benzanthracene) The carcinogen is administered as a single dose to female rats at the age of one and a half to two months and tumours can start appearing and physically palpable after 3 to 4 weeks post-administration. The anticancer test drug is administered at the appropriate tumour volume, and change in tumour volume is recorded to measure drug efficacy. The detailed histopathological and immunohistochemistry examination of tumour is valuable in determining the drug action (Nicol et al. 2004).

Colon Cancers Induced Using DMAB (3, 2-dimethyl-4-aminobiphenyl) These tumours appear after subcutaneous injection of the carcinogen is administered for consecutive 20 weeks on a weekly basis. It induces multiple colon tumours in about 26–30% of animals fed on modified fatty diet. The tumours induced are adenomas (benign tumours) as well as adenocarcinomas (malignant tumours) in the large bowel. This model gives good information on tumour inductions and shape, size and histological type of tumour tissue. Only drawback with this model is requirement of multiple carcinogen injections and formation of secondary tumours at other sites such as the skin, mammary glands, stomach, salivary glands, lymph nodes, ear canal and urinary bladder. This influences and complicates the comparison of drug efficacy and survival studies (Nicol et al. 2004).

Fibrosarcoma Model in Mouse Using 3,4,9,10-dibenzopyrene Single subcutaneous injection of carcinogen is sufficient to form uniform subcutaneous tumours at the site of injection within 30–40 days post injection. The most important advantage of this model is induction of tumour with single dose of carcinogen and no excretion of carcinogen through faeces or urine (Nicol et al. 2004).

Virus-Induced Tumour Models Peyton Rous (Nobel Prize, 1966) from Rockefeller University showed viral aetiology of cancer in chickens in 1911 and opened up the field of oncoviruses. This oncovirus is named after him as Rous sarcoma virus (RSV) for his significant contribution in cancer aetiology. The Jackson Laboratory also identified mouse mammary tumour virus (MMTV) in the C3H strain of mice that caused mammary tumours. The transmission of this virus is through milk from mothers to pups (Golovkina et al. 1998).

There are viruses which produce cancer via random integration in certain cells; also some carry cellular oncogenes such as Abelson murine leukaemia virus (Abl), Moloney murine sarcoma virus (Raf), FBJ osteosarcoma virus (Fos) and Friend murine leukaemia virus (c-fms).

9.2 Genetically Engineered Mouse (GEM) Models

Cancer originates from changes in genes, and hence the animals genetically engineered depict cancer scenario in humans better than other animal models. In these animals, there is a spontaneous development of cancer in its natural anatomical site, unlike subcutaneous xenograft tumour models. In GEM, tumours show natural progression pattern and metastatic biology similar to human counterpart. Importantly these tumours are originated within self; hence, there is no need for immunosuppressed animal to grow them.

GEM animals include transgenic and knockout mice.

9.2.1 Transgenic Mouse Model

These animals are born as a result of insertion of exotic gene in the pronucleus after fertilization of egg. The subsequent progeny developed from this modified egg carries and expresses the exotic gene which was inserted and also passes it further to its descendants. The gene to be studied can be introduced into the pronucleus by microinjection, retroviral vector or embryonic stem cell (ESC) transfer. This proves to be an excellent animal model system to study oncogenic phenotype resulted from the dysregulation of a known gene (Abate-Shen and Shen 2002).

One can study the characteristics of oncogenes in these animals such as TP53, c-Myc, E2F1, RB, NF1 and others. These oncogene-expressing animals develop spontaneous tumours due to a known pathway defect and therefore are most suitable for evaluation of drugs/molecules specifically targeted for those molecular pathways in transgenic mouse models (Abate-Shen and Shen 2002; Hursting et al. 2005).

9.2.2 Knockout Mouse Model

Basically, in these animals, the gene is deleted or inactivated artificially using synthetic DNA segment. These knockout mice are the other type of genetically engineered animals generated by deleting both the alleles of a gene of interest, e.g. Nkx 3.1 knockout mice. NKX 3.1 is a tumor suppressor gene plays an important role in normal prostate development, regulation of glandular epithelium proliferation and in the formation of prostatic ducts. This mutation is observed in 60–80% of prostate malignancy. The initiation, progression and metastatic behaviour of cancer in these animals are very similar to human counterpart; hence, this model provides unique opportunity for the study of all these steps in prostate cancer development (Wu et al. 2013). This method has been explored for developing models for other cancer types.

There are several GEM models available for cancer research and anticancer drug screening. The number of these models has increased with the understanding of molecular pathways involved in cancer and advent of newer molecular technologies.

9.3 Transplantable Tumour Models

These models are most commonly used model system utilizing cancer cell lines or tissues of human or mouse origin. These models are divided into two types depending on methods of transplantation.

9.3.1 Heterotopic Tumour Transplantation

In this case, implantation of tumour cells or tissues is done at a site other than its anatomical site. For example, breast cancer cells are transplanted subcutaneously or intraperitoneally. This method generally involves subcutaneous or intraperitoneal transplantation of tumour cells; the tumour proliferates in that site to form solid nodules or ascites, respectively. The procedure of inoculation is simple, quick and precise and allows inoculation in a large number of animals for screening of anticancer agents (Khanna and Hunter 2005).

Orthotopic Tumour Transplantation The procedure of this is different from that of the heterotopic model as it involves inoculation of the cancer cells into the original anatomical location from which the tumour is. For example, mammary tumour is transplanted in mammary fat pad in female mouse. This method has more close similarity to human cancers, including tumour stroma, which mainly contains basement membrane, fibroblast, extracellular matrix besides the histological type, vascular network, genetic profile, response to anticancer therapies and metastatic pattern. As we understand more about host-microenvironment interaction in tumour initiation, progression and metastatic behaviour, it is clear that orthotopic tumour models are superior over conventional flank mouse models.

These models are developed by inoculation of cancer cells by direct injection or by surgical procedure in anatomical site, i.e. implantation of the cancer cells or intact fragments of tumour orthotopically by invasive procedure. Surgical implantation is invasive as it improves the reproducibility and metastatic behaviour of the model (Khanna and Hunter 2005). The post-operative care in these animals is important for their recovery.

Transplantable tumour models are further categorized into two depending upon the origin of the tumour and the host used.

9.3.2 Syngeneic Mouse Models

In these animal models, the cancer cell line or tissues of murine origin are used for transplantation. The transplantation procedure is performed in the animals of genetic homozygosity as they are derived from same cell line or tissue. For example, B16-F10 melanoma cell line obtained from C57BL/6J mouse is used to develop cancer models in animals of same species. These tumours are basically carcinogen induced

or spontaneous in nature, which are collected and maintained as cell lines. The important benefit of this model system is the similarity of species of host to transplanted tumour tissue. This allows acceptance of these cells or tissue without any immunological interference. But in this model system, important characteristics of human tumours are lacking such as genetic heterogeneity and species-specific differences in oncogenesis (Voskoglou-Nomikos et al. 2003). These animals are useful in studying tumour biology and metastatic behaviour besides their ease of development and maintenance.

9.3.3 Xenograft Models

These tumour models are developed using transplantable tumours of human origin and hence more closely resemble the clinical scenario. As the implanted cancer cells are from different host, i.e. human tumours in mice, it results in severe immunological reaction rejecting the graft. To overcome this hurdle, the immune system of these animals is suppressed by using immunosuppressive agents or by thymectomy i.e. surgical excision of thymus or irradiation of animals (Khleif and Curt 2000). However, these methods are cumbersome, less reliable and not feasible in a large number of animals. Hence use of immunocompromised animals such as athymic (nude) mice and severe combined immunodeficient (SCID) mice was accepted for this purpose. These animals lack immune cells and hence do not react to the foreign transplanted material.

The first nude mouse was found naturally in a closed colony of albino mice in a Ruchill Hospital laboratory in Glasgow, Scotland, by Isaacson and Cattanach. These mice were characterized phenotypically as the ones lacking haircoat (Rygaard and Poulsen 1969). On the 11th chromosome of these animals, a mutant gene (*nu*, for nude) is present as an autosomal recessive gene, which is responsible for the lack of hair, shunted growth, short lifespan and reduced fertility. These animals with homozygous mutation *nu*/*nu* lack a thymus, while the thymus is present in heterozygous nu/1 animals. Immunologically, athymic mice have less number of T cells, which are received from heterozygous mother. However, the B cell function in these animals is normal with higher natural killer cell activity. Successive passages of human tumour xenografted into nude mice and its ability to maintain their histologic and biologic identity revolutionized many aspects of cancer research. Transplantation of tumour cell lines into immunocompromised animals can be accomplished via multiple routes such as subcutaneous, intraperitoneal, intravenous, intracranial, intrasplenic and renal subcapsular or through a new orthotopic model by site-specific organ inoculation. Each site has specific advantages and limitations. During this procedure, tumour cells undergo kinetic changes, and often their doubling time becomes shorter than that of the original tumour, which further decrease during subsequent in vivo passages. Despite this, xenografted human tumours maintain many original morphologic and biochemical changes. Therefore, human tumour xenografts are mainstay of cancer drug discovery programmes (Tentler et al. 2012).

Newly developed mouse models with improved immune alterations have increased use of these model systems for basic and translational cancer research. Previously, establishing human xenograft tumours for different cancers was very time consuming and challenging but with availability of immunocompromised animals such as nude, SCID, beige and xid it became easier (Liu and Hicklin 2011).

Further, mice having two gene mutations such as NOD/SCID-IL-2g null (NSG), NOD/SCID-IL-2g null + human cytokine expression and NOD-Rag-IL-2g null (NRG) have progress in developing 'humanized' mouse by bone marrow cells or human peripheral blood injection. This intervention permits reconstitution of immune response to the tumour. Besides new techniques and procedures for implantation and engraftments of tumour were developed to refine human relevance of these models. Many researchers started using orthotopic xenograft techniques, which closely mimic the human tumour microenvironment (Richmond and Su 2008).

Though there are huge refinements in mouse model systems, still new changes are being faced in answering specific research questions in cancer biology as well as developing treatment strategies using these models. Despite these improvements, the importance of xenograft models is still under study. Retrospective data generated from these models revealed failure of compounds in human clinical trials in spite of their promising activity in xenograft models because of limited efficacy or high toxicity (Suggitt and Bibby 2005).

Cell line-based xenografts which are maintained in vitro for longer period do not represent original tumour due to deviation from original characteristics at molecular level (Suggitt and Bibby 2005). Hence there are differences in demonstrating drug response in cancer patients (Jung 2014). Presently considerable information is available about xenograft models hence rational matching of model with the specific molecular drug target is essential. To avoid these drawbacks of cell line models, scientists are focusing on patient-derived xenograft (PDX) models as a promising solution in the anticancer drug development process (Jung 2014).

9.4 Limitations of Using Immunodeficient Mice

The complex nature of cancer due to its heterogeneous tumour microenvironments is not represented well in immunocompromised animals. Even though these tumours can easily grow in mice, there is possibility of insertion of mouse specific attributes in the tumour microenvironment such as stroma and vascular activators during serial passaging hence implanted mouse tumour looses its similarity with original human cells. Besides, there are species-specific differences between human and murine hosts in terms of drug metabolism, influencing differences in activity and levels of drug. The drug dosing in mice often requires modulations to achieve blood levels observed in humans. Precise representation of tumour microenvironments is necessary while testing compounds targeting tumour environment factors such as anti-angiogenesis components, hormone antagonists and immune checkpoint inhibitors.

The anticancer immunity and graft-versus-host immunity are not completely disrupted in these models. Some human tumour xenografts fail to optimally grow in current immunodeficient models because of innate immunity, which is not completely disrupted (Brehm et al. 2010).

There is limited utility of these animals for immunotherapy studies such as blocking immune inhibitory receptors or directly stimulating the immune system (Sharma and Allison 2015). The evaluation of immune-modulating drugs or antitumour vaccine therapies which target the specific human proteins in immunodeficient mice is not feasible. Hence development of various types of humanized mice has been envisaged.

Host immune system regulates tumour growth and metastasis. Both macrophages and B lymphocytes promote angiogenesis as well as lymph angiogenesis influencing tumour progression and metastasis. Accordingly PDX tumours developed in immunocompromised animals may show differences in vascular and lymphatic infiltration impacting tumour proliferation (Qian and Pollard 2010; Ruddell et al. 2003).

In immunodeficient animals the growth and development of secondary lymphoid organs, such as lymph nodes, is severely impaired. They either have very small lymph nodes or lack some of the group of lymph nodes (Brehm et al. 2013), thus altering the natural path of the haematopoietic cancers in these models. Besides, there are differences in cell adhesion molecules in humans and mouse, viz. integrins and selectins, which influences natural migration and retention of specific human cancer cells in murine hosts.

There are human cancers associated with pathogens such as viruses, bacteria and parasites. Immunocompromised animals are not suitable for cancers which are associated with microorganism as host immune system plays a pivotal role in cancer initiation and progression.

Engraftment rates for some tumours remain low and hence its establishment can be lengthy. Ideally, during initial treatment of cancer patients, PDX mice could play an important role in assessment of therapeutic response to specific drugs. However, some tumours require many months to be formed with inconsistent engraftment rates; thus, availability of large group of animals for testing purpose is not feasible. Furthermore the cost of PDX studies is still very high making it not so easy option.

9.5 Patient-Derived Xenograft (PDX) Tumour Model

Xenograft mouse models have several positive points, but they have limitations in their capacity to predict drug response in cancer patients. The accurate prediction of drug response is important in a clinical trial, which is not possible with previously discussed animal model systems. PDX system has been developed to provide solutions for limitations seen in existing models (Tentler et al. 2012; Moro et al. 2012). As in PDX model, there is transplantation of tumour tissue from patient into immunodeficient animals; the tumour genetics and immunohistological markers are

directly correlating to the cancer patient. Hence these models can be used to develop personalized cancer therapies (Fichtner et al. 2008).

However, this PDX system has few constraints such as cost and time required in development of the model. In this scenario the freshly surgically excised tumour tissue from patient needs its transfer to vivarium from the operation theatre within few hours. The sample from patients should be carefully seen and checked by immunohistological analyses. Hence requires close coordination among multiple experts such as surgeons, pathologists, scientists and laboratory personnel for proper authentic sampling of tissue as well as its further passage in model system. Prior to these procedures, approvals from human and animal institutional review board for clinical and ethical considerations are necessary. The engraftment rate of PDX model is about 25% in spite of following all measures carefully (Zhuo et al. 2010; Jin et al. 2011; Bertotti et al. 2011). It almost takes 3 months to establish the first passage. The tumour tissue is very small in volume; hence generation of large number of model animals is challenging. Besides, at every passage, assessment of tumour tissue with primary tumour is important to assure there is no deviation. To control cost nude mice can be used from the second passage onwards, and tumour tissues are stored in liquid nitrogen for assessment. PDX model has been explored globally as it shows promise for personalized cancer therapy.

9.6 Methodology for Generating and Validating the PDX Model

During PDX development cancerous tissues are implanted from a cancer patient directly into an immunocompromised animal (Fig. 9.2). The group of immunocompromised animals utilized in developing PDX models are athymic nude mice, severe combined immunodeficient (SCID) mice, nonobese diabetic (NOD) SCID mice, etc. (Morton and Houghton 2007).

Single-cell suspension and tissue fragment are two different input materials that can be used for the PDX generation. The tumour tissue is acquired after surgical procedure is performed in cancer patients. The appropriate cancer tissue is taken to the lab in proper culture medium, i.e. Dulbecco's modified Eagle's medium [DMEM] or RPMI with 10% foetal bovine serum [FBS] and 1% penicillin/streptomycin. The tumour tissue is cleaned by removing areas of necrosis and further divided into small fragments. Then single-cell suspension is prepared by chemical digestion or physical methods. One can use smaller fragments or single-cell suspension; both methods can very well be used considering their advantages and disadvantages carefully. Tumour fragments represent tumour microenvironment of the original tumour, and in case of single-cell suspension, the proper tumour representative cells can be collected in a more unbiased manner (Williams et al. 2013). While making single-cell suspensions, these cells are prone to strong chemical or mechanical treatment leading to changes influencing cell viability and further engraftment (Zvibel et al. 2002).

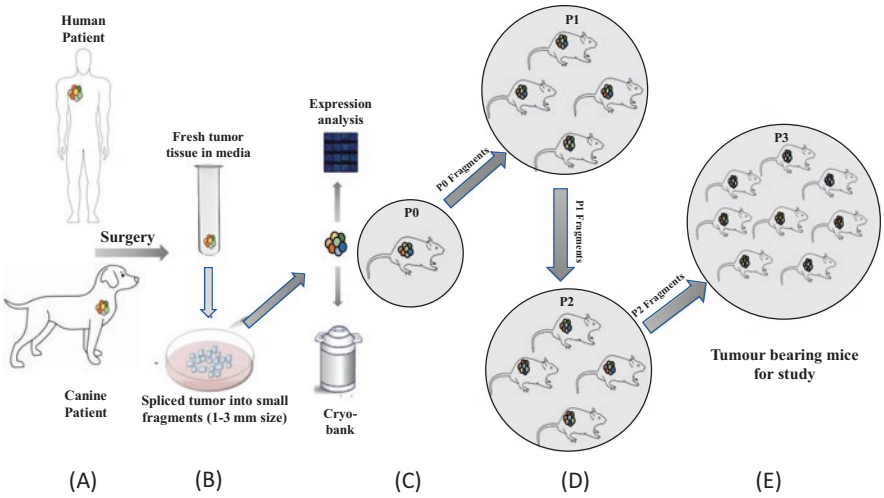

Fig. 9.2 An overall procedure of establishment of PDX model. Cancer tissues from human and canine patients are recovered after surgery (**a**). Tumour tissue is collected in freezing media and spliced into small fragments of 1–3 mm for implantation (**b**). Small tumour fragments are implanted in mice and also stored in cryobank for expression analysis study (**c**). Tissues are further passaged as P1, P2 (**d**). Final passage animals are used for further studies (**e**)

The tumour fragments or cell suspension is implanted in the recipient mouse either heterotopically or orthotopically. In heterotopical implantation procedure, the tumour fragment is implanted subcutaneously or in subrenal capsular sites in mouse which is different from the original tumour anatomical site.

In case of orthotopic tumour transplantation, tumor tissue is transplanted into the anatomical site of primary tumour as described in earlier section. Similar to cell line-based subcutaneous xenograft model, subcutaneous PDX also has inability to metastasize and depict tumour microenvironment accurately with primary tumour (Zvibel et al. 2002). Orthotopic models are ideal models, which mimic the natural microenvironment of primary tumour, but are difficult to generate due to their organ specificity (Hanahan and Weinberg 2011). The time taken for engraftment of tumour in mouse is between 2 and 4 months and depends on various factors such as type of tumour, location of implantation, the mouse strain used in development of model, size and number of tumour fragments used for implantation, number of cells and medium used in case of single-cell suspension inoculation, etc. Considering these several factors, the failure of engraftment should be observed maximum for 6 months before indicating as unsuccessful event (Morton and Houghton 2007).

The harvested tumour tissue with small fragments of 1–2 mm^3 needs to be washed 4–5 times using culture medium and then after addition of freezing medium along with FBS and 10% dimethyl sulphoxide should be stored at −80 °C for future utility such as transplantations into other animals later. The first generation of animals which receive tumour fragments directly from the patient is denoted by *F0*. When tumour has grown to a desired size, it can be dissected well and used further for the next generation, i.e. F1 recipient animals. The future generations are notified

Table 9.2 PDTX engraftment rate for different tumour types and different anatomical sites utilizing various mouse strains

Tumour type	References	Mouse strain	Implantation site	Engraftment rate (%)
Colorectal cancer	Ma et al. (2011)	NOD/SCID	s.c.	87
Colorectal cancer	Nicol et al. (2004)	Nude	s.c.	64
Colorectal cancer	Kinosita (1936)	Nude	Orthotopic	89
Breast cancer	Winograd et al. (1987)	Nude	s.c.	13
Breast cancer	Filipiak and Saunders (2006)	NOD/SCID with oestrogen supplementation for ER^+ tumours	Mammary fat pad	27
NSCLC	Winograd et al. (1987)	NOD/SCID	s.c.	25
Pancreatic ductal carcinoma	Weisburger and Weisburger (1958)	Nude	Orthotopic	62
NSCLC	NCI Dtp Drt (2015)	NOD/SCID	Renal capsule	90
SCCHN	Plowman et al. (1997)	NSG	s.c.	85
Pancreatic ductal carcinoma	Wolf et al. (2000)	Nude	Orthotopic	62
SCCHN/SCC	DeVita and Chu (2008)	Nude	s.c.	54
Uveal melanoma	Winograd et al. (1987)	NOD/SCID	s.c.	28
Gastric cancer	Hammer et al. (1985)	Nude and NOG	i.p.	31
Prostate cancer	Voskoglou-Nomikos et al. (2003)	NOD/SCID	Subrenal capsule	95
Renal cell carcinoma	Boven et al. (1992)	Nude	s.c.	8.9

as F1, F2, F3 and so on. If necessary the tumour stored in −80 °C can be used for further passaging by thawing completely in 37 °C water bath, and then it can be transferred to a medium containing plastic tube. After its complete thawing, the fragments can be used for reimplantation in next batches of animals (Jin et al. 2010).

Nowadays PDX models are available commercially for various human cancers. On the basis of previous studies conducted by several researchers in the past, the success rate of various human cancer types along with specific engraftment methods used has been recapitulated in Table 9.2 (Fichtner et al. 2008; Bertotti et al. 2011; Julien et al. 2012; Aytes et al. 2012; Marangoni et al. 2007; DeRose et al.

2011; Li et al. 2013; Garrido-Laguna et al. 2011; Reyes et al. 1996; Kimple et al. 2013; Keysar et al. 2013; Némati et al. 2010; Choi et al. 2016; Boone et al. 2015; Sun et al. 2010).

There are several factors that influence the success rates of PDX such as nature of primary tumour with respect to its invasiveness, tissue type and number of malignant cells in the tumour tissue. For example, breast cancer with triple negative status, i.e. cells lacking ER (oestrogen receptor), PR (progesterone receptor) and hEGFR-2 (human epidermal growth factor receptor 2), has better engraftment as opposed to cells with positive receptor status (Tentler et al. 2012).

Other factors such as PDX methodology also influence the success rate. The tumour tissue to be used for PDX should be collected quickly as fresh as possible, reducing the time from surgical excision till the processing in laboratory. The use of freshly prepared medium is recommended to store tumour tissue after its collection during surgical procedure. The outcome of engraftment is significantly influenced by number and size of tumour tissue fragment to be implanted. More number of fragments improves the success rate. For better outcome, optimal fragment size preferred is 1–2 mm^3. The anatomical location of fragment implantation is also important. It has been reported that subrenal capsule implantation has better engraftment rate than subcutaneous implantation, as subrenal capsule provides microenvironment equivalent to host stroma (Marangoni and Poupon 2014). Although the ability of the tumour tissue to embrace the host environment depends on original tumour, the tumour grows significantly well in immunocompromised animals.

PDX Model Validation and Molecular Characterization The harvesting of tumour is done after it grows to a desired size of 1–2 cm in diameter. Mouse tissue with tumour should be harvested properly and a part of tumour should be preserved in 10% formalin. The formalin-fixed paraffin-embedded (FFPE) tissue blocks are prepared and further used for validation of tumour type and immunohistochemical markers in original patient tumour as well as passaged tumour tissue.

Whole-gene sequencing and cancer panel can be done from genomic DNA extracted from frozen tissue. RNA purification would be done for further RNA sequencing to evaluate gene expressions. For determination of protein expression, PDX tissue from primary tumour as well as passaged tumour can be used directly.

9.7 Canine Model for Cancer Research and Preclinical Drug Testing

The efforts in search of relevant mouse model led towards comparative oncology approach for understanding cancer biology and pathogenesis and developing novel therapeutics (Sultan and Ganaie 2018; Withrow et al. 2013). In the past two decades, comparative oncology has grown to a significant mark and is presently seen as the most promising approach to deal with cancer (Parrales et al. 2018; Hansen and Khanna 2004; Paoloni and Khanna 2008) (Fig. 9.3). Comparative oncology is a

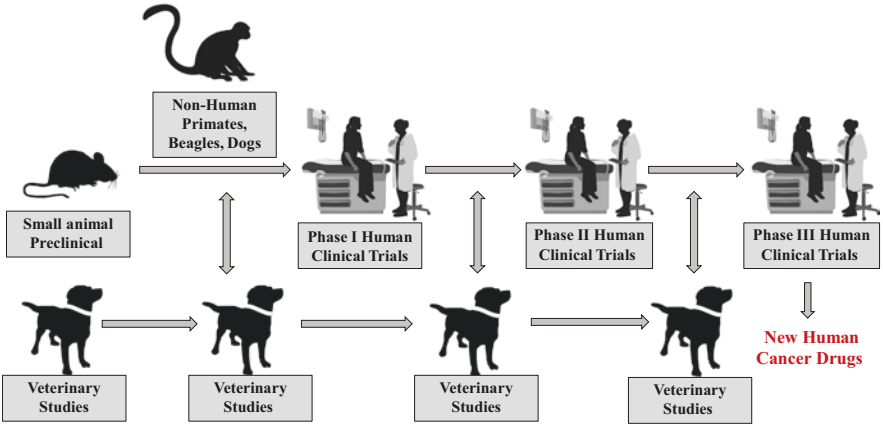

Fig. 9.3 A clinical trial design showing use of laboratory animals for human cancer research. Similarly, spontaneous canine cancer models can be used for new anticancer drug development

discipline that deals with incorporation of the spontaneous cancers in animals such as dogs into cancer research and the subsequent development of novel therapeutics (Hernandez et al. 2018; Segaoula et al. 2018). Other veterinary patients such as cat, cattle and horses often get cancers and are of comparative interest as well; however their integration in new anticancer drug trials is not feasible for several reasons. One of the most important reasons being they are not treated as humans when they get cancer. In case of canine patients, the diagnostic workup, treatment regimes and combinations of drugs are often comparable. Besides, the pet parents share a social and emotional bonding, which lead them to get the best treatment available for their ailing pets.

The comparative interest in this model is studying environmental associations, epidemiology of cancers on the basis of nutritional patterns, involvement of cancer-associated genes and proteins, genetic susceptibility of breeds and age-related cancer occurrences (Pinello et al. 2019). This model has huge potential because of similarities with human cancers in anatomical and physiological characteristics, cancer cell types and morphology, metastatic behaviour and age dependency of cancer occurrence (Schiffman and Breen 2015) (Fig. 9.4). The most common cancers seen in pet dogs are mammary, cutaneous, bone, oral and bladder and lymphoma (Baioni et al. 2017). The reporting of cancer in pet dogs has increased over the years mainly due to awareness among pet parents, availability of diagnostic facilities and change in attitude towards dogs from a pet to a companion. The comparative oncology-focused clinical trial designs have been proposed and are currently in use at several academic institutes having full-fledged comparative oncology programme.

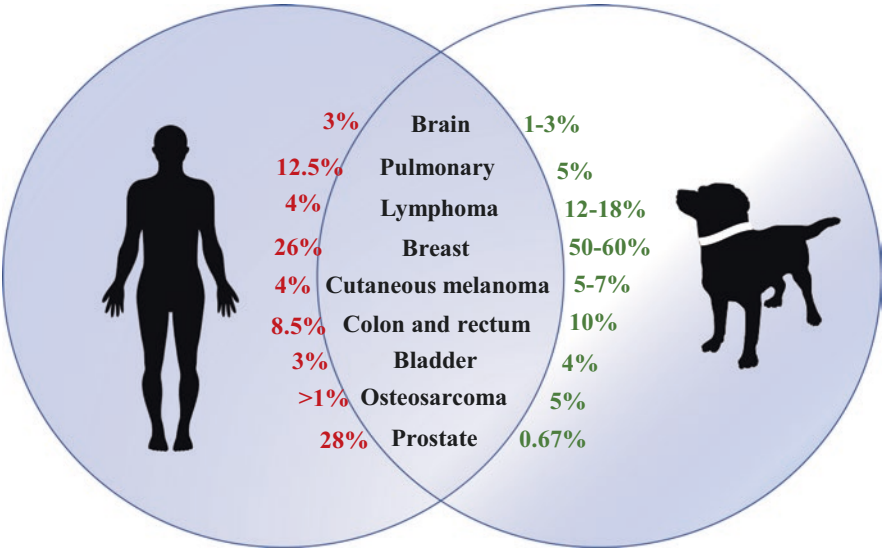

Fig. 9.4 Comparative incidence of organ-wise cancer occurrence in human and canine patients

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Dysregulated Signaling Pathways in Cancer: Approaches and Applications

10

Pranay Ramteke, Dipti Athavale, and Manoj Kumar Bhat

Abstract

Cancer-related deaths account for more than 8.8 million individuals per year globally. In spite of exhaustive literature about dysregulated signaling pathways in cancer, correlating it to patient survival warrants further understanding. Therefore, it is pertinent to investigate the working principles behind cellular and molecular tools which facilitate in delineating aberrant alterations used for targeting cancer cells. In addition to existing cell biology techniques, immunoblotting, PCR, FACS, ChIP, EMSA, etc. have immensely contributed to decipher altered molecular and metabolic pathways in cancer. Collectively, these have paved a way toward discoveries and inventions on which modern-day diagnostics and therapeutics are based. This chapter summarizes important techniques in molecular biology and the differential characteristics they target, to understand the approaches used to detect, diagnose, and treat cancer.

Keywords

Dysregulated signaling pathways · Immunoblotting · Reverse transcription polymerase chain reaction · DNA-protein interactions · Cancer therapeutics

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10.1 Introduction

Cancer is a fatal disease caused due to uncontrolled proliferation of cells accounting for the second highest mortality after cardiovascular diseases, across the globe (8.8 million deaths in 2015). Around the world, approximately one in six deaths occurs due to cancer. Despite centuries of consistent research, a cure for cancer is not yet discovered. Although many therapeutic advances have emerged since the very first report of the disease, which target various stages, grades, and hallmarks of cancer, a specific cure is still not in vision. This makes cancer even more of a deadlier disease than cardiovascular diseases as much of the mortality due to the latter is due to aging and organ failure, eventually leading to death (Ferlay et al. 2013; http://www.who.int/news-room/fact-sheets/detail/cancer, 2018). Hence, there is a paramount need of an in-depth understanding, analysis, and study of cancer and its molecular and metabolic reprogramming along with the tools and techniques that are available. With the knowledge of how these cellular/molecular tools and techniques contribute toward its diagnosis and therapeutics, a better understanding and future directions in cancer research need to be chalked out in order for proper management of this lethal disease.

- *History of Cancer and Oncogenesis*

Cancer is known to exist even before the pre-human era itself. Evidence of cancer cells was found in dinosaurs which date back to 70–80 million years ago. The first report of human cancer appeared in the scriptings of Edwin Smith Papyrus roll and Ebers Papyrus. This first generation of cancer scientists defined and coined most of the terminologies used in modern era of cancer. They used a variety of strategies and medications for treatment of cancers, some of which laid the foundations of currently existing treatments like advanced surgeries, whereas others have been outdated with time and the advent of science and technology as described in Table 10.1 (Di Lonardo et al. 2015; Lukong 2017; Felipe et al. 2017).

The modern-day understanding of cancer derives from extensive investigations of all known characteristics of the disease which were described throughout the literature by cancer biology scientists. One of the most quintessential descriptions of cancer and its related phenomena was mentioned as the hallmarks of cancer as described in Fig. 10.1 (Todd et al. 1884; Hanahan and Weinberg 2011).

- *Oncogene and Cancer Cell Metabolism*

Activation of oncogenes or loss of tumor suppressors leads to cancer occurrence and cancer progression (Vogelstein et al. 2004). These genetic alterations can rewire cancer cell metabolism in such a way that it supports rapid cell growth and proliferation. For instance, to provide the energy for growth, cancer cell uses a high flux

Table 10.1 Milestones in cellular and molecular cancer biology

The very first occurrences, descriptions and milestones of cancer research	The major advancements in cancer research from the past
Paleolithic era: 70 – 80 million years ago Tumors in prehistoric animals Evidence of cancer cells in dinosaur fossils, found in 2003.	81-138 Aretaeus proposes the first description & treatment of uterine cancer.
	100-200 Galen hypothesizes that thick black bile leads to an incurable form of cancer, and that cancer caused by thin yellow bile is curable. He also uses the Greek word "oncos" i.e. swelling to describe tumor.
4.2 – 3.9 million years ago Louis Leakey in 1932 found the oldest known hominid malignant tumor in Homo erectus, or Australopithecus.	1500s Paracelsus proposes that accumulation of harmful substances in the bloodstream may lead to cancer. Also, A. Pare proposes cancer may be caused due to diet irregularities that induce accumulation of feculent blood material.
3000 BC: Edwin Smith Papyrus : First written description of cancer e.g. : Case 39 about breast cancer	1800s The discovery and advent of microscopes and implementation of histological techniques helps the detection as well as analysis of anomalous cell nuclei in cancer cells.
	1860, R. Virchow describes cancer as disease of cells.
	1891, Hanseman hypothesizes *'The cell of the malignant tumor is a cell with a certain abnormal chromatin content'*.
1500 BC: Ebers Papyrus describing stomach, skin, rectum cancer.	1908, Ellerman and Bang describe the causative agent of leukemia is a virus.
	1956 Otto Warburg shows the altered glucose metabolism which is exhibited by cancer cells.
400 BC: A greek physician Hippocrates-,also known as the 'father of medicine', coined the term cancer derived from the word 'karkinos" (Greek means crab or crayfish)	1969, Virogene/oncogene hypothesis by R. Huebner and G. Todaro
	1973, J. Rowley shows chromosome abnormalities in patient with cancer.
	1994, BRCA1 & BRCA2 identified as anti-oncogenes from breast cancers.
25-50 BC: Aulus Celsus in his sbook De Medicina described different types of cancers. Also hypothesized that breast cancer can lead to death by migrating to other organs, & surgical therapy should be implemented.	2008, first whole cancer genome was sequenced from cytogenetic Acute Myeloid Leukemia (AML), compared with normal somatic skin cell from the same individual.

of glucose and synthesizes ATP with faster rate via glycolysis, also known as Warburg effect (Russell et al. 2009). Additionally, intermediates of glycolysis generate redox equivalents which are utilized in macromolecule (nucleotides, lipid) biosynthesis. For sustained glucose supply, cancer cells often overexpress glucose transporters (GLUTs). This overexpression is correlated with oncogenes like c-Myc, Ras/Raf, etc. Apart from Glut1 regulation, MYC oncogene transcriptionally regulates enzymes involved in glucose metabolism. LDH-A (lactate dehydrogenase A) which converts pyruvate generated by glycolysis to lactic acid is induced by c-Myc. Another oncogene, Akt, is involved in activation of nutrient transporters, glycolysis, and lipid metabolism in transformed cells. It also induces mTOR (mammalian target of rapamycin) pathway, a signaling cascade involved in protein translation which is essential for tumor growth. Owing to uneven vasculature, cancer cells often have to face metabolic stress like nutrient scarcity. To overcome, AMPK (AMP-activated protein kinase), an energy sensor regulates cellular proliferation in nutrient-deficient conditions. Although AMPK is considered as tumor suppressor, recent reports imply its role in adapting tumor cells toward low nutrient availability, thus its involvement in their survival (Chaube et al. 2015). In the current chapter, various metabolic and molecular approaches used to dissect differential metabolic pathways and their regulation in cancer cells have been discussed.

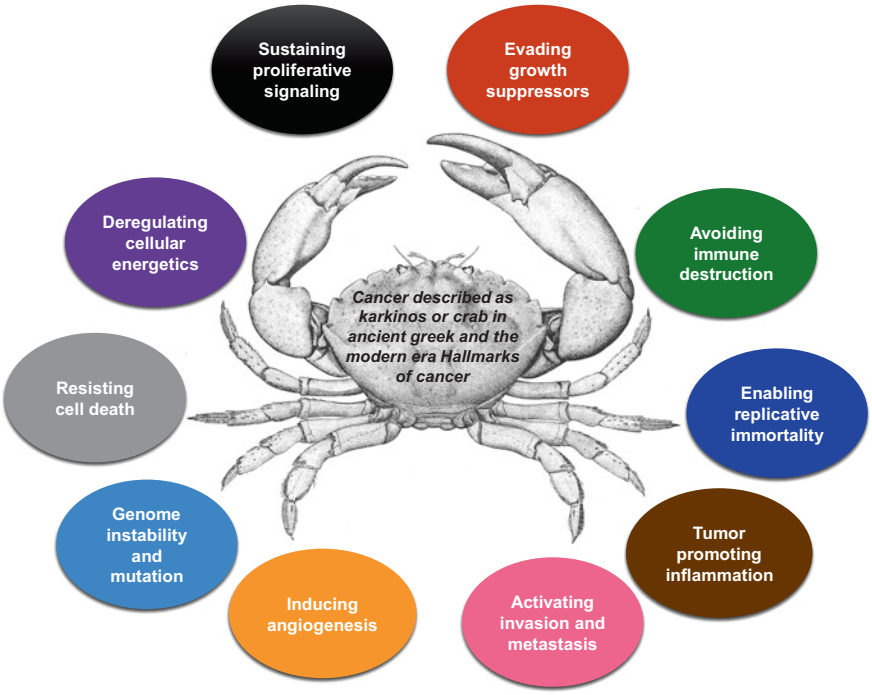

Fig. 10.1 Amalgamation of ancient and modern-day perspectives in cancer. Cancer was described as "Karkinos" or crab in ancient times due to the tumor and its swollen blood vessels around it in a local tissue. Centuries after the first description, modern-day scientists described various hallmarks of cancer or the various characteristics exhibited by cancer cells which can be imagined as the appendages of the crab which helps it to adapt, penetrate, survive, dominate, spread, hijack, and eventually kill the host

10.2 Molecular Techniques to Study Cancer Metabolism

- *Western Blotting/Immunoblotting*

Principle Immunoblotting is one of the most important and commonly used techniques in molecular biology. The objective of an immunoblot is to detect single or multiple protein(s) from a mixture of proteins (usually detecting the protein of interest from whole cell lysate of proteins from cells or tissues), by separating them based on respective molecular weights, and detect with the help of an antibody.

Methodology Immunoblotting is usually performed in three steps: PAGE, transfer, and detection.

1. *PAGE (polyacrylamide gel electrophoresis):* PAGE is the electrophoretic separation of proteins on a polyacrylamide gel based on respective molecular weights of the proteins. Equal amounts of sample (lysates or purified protein) are mixed with loading dye consisting of a negative charge-imparting agent (SDS (sodium dodecyl sulfate)), chemical bond-cleaving reagents (e.g., β-mercaptoethanol), a density-imparting agent (sucrose or glycerol), and a dye such as bromophenol blue for index run and heated at high temperature for denaturation. These samples are loaded in PAGE wells and are subjected to electrophoresis by applying constant voltage across the gel with the help of electrodes. The charged proteins are hence separated on the basis of their respective molecular weights as proteins with different molecular weights migrate at different velocities toward the oppositely charged electrode.
2. *Membrane transfer:* Transfer of the separated proteins onto a PVDF (polyvinylidene difluoride) or nitrocellulose membrane for further detection. The gel is kept in direct contact with the membrane in a sandwich of transfer pads and filter papers. Constant electric current is applied in presence of transfer buffer to facilitate transfer of these proteins onto the membrane with help of hydrophobic interactions and charge.
3. *Detection*: After transfer of proteins separated on the basis of their molecular weights onto a membrane, the protein of interest can be detected by probing the membrane with commercially available antibody raised against the protein of interest. The protein-antibody complex is probed with a secondary antibody preconjugated with an enzyme (HRP or ALP) or fluorochrome which can be detected by chemiluminescent/fluorescence methods, e.g., autoradiography on x-ray film by adding the substrate of the enzyme. This enzymatic reaction gives luminescence in dark in the form of separated bands where the primary antibody against that particular protein of interest is bound on the membrane. The size of this band is directly proportional to the abundance of protein in that particular sample and can be compared with other samples run alongside or differently in adjoining lanes of the same gel. In the case of fluorochrome-tagged antibodies, direct exposure in fluorochrome detecting devices can be performed. If an antibody is not present commercially, the separated protein can be subjected to mass spectrometry analysis for identification by cutting the band of interest from gel and isolation of the protein from cut gel.

Applications Immunoblotting can be used to study various aspects of protein biology:

1. Measuring relative protein abundance.
2. PTM (protein posttranslational modifications), e.g., glycosylation, acetylation, phosphorylation, ubiquitination, sumoylation, etc., by using phospho-specific antibodies or other modification-specific antibodies.
3. Protein-protein interactions by using Co-IP (co-immunoprecipitation).

4. Cellular localization by using cytosolic, membrane, and nuclear fractionation.
5. The changes in protein levels often infer to the function carried out by them and therefore the phenomena occurring.

Since proteins are the functional units of cells which regulate and carry out various phenomena and cellular signaling in cancer is often dysregulated, detection of these altered pathways, i.e., altered levels of proteins, is of prime significance in a cancer study. Therefore, Western blotting is often found at the heart of any cancer research.

For example, a protein is hypothesized to up-/downregulate under specific conditions, and this needs to be proven experimentally. In this scenario, a Western blot is performed for detection of altered levels of this protein by culturing cells in the hypothesized conditions and normal (control) conditions. The lysates of cells cultured in these conditions are run in separate wells alongside each other in the gel and subjected to transfer and further detection. Bands are obtained by above mentioned methods. Comparing levels of this protein band in the control/untreated condition with treated (hypothesized) condition clearly indicates whether there is any effect of the hypothesized treatment/condition on the levels of that protein and therefore the function carried out by that protein. PTM levels and cytosolic and nuclear levels can also be checked similarly (Bass et al. 2017; Jensen 2012; Protein Blotting Guide 2018).

- *RT-PCR (Reverse Transcription Polymerase Chain Reaction)*

RT-PCR enables quantification of messenger RNA (mRNA) transcripts and provides information about gene expression. Apart from RT-PCR, Northern blot analysis and RNase protection assays are used to quantify mRNA levels. RT-PCR technology consists of two steps: the first step in which complementary DNA (cDNA) is synthesized from mRNA molecules by reverse transcriptase enzymatic reaction and the second step in which newly synthesized cDNA is amplified by PCR.

1. *Reverse Transcription (RT)* – Total RNA is isolated from multiple biological replicates for statistical analysis of the data. RNA quality is checked by spectrophotometer (A260/280 > 1.8, A260/230 > 2.0). A_{260} values define the quantity of total RNA. Purified RNA can be digested with DNAse I to remove genomic DNA contamination. RT reactions are performed using reverse transcriptase and either random primers or oligo-dT (Pittman 1996).
2. *Polymerase Chain Reaction (PCR)* – This step of RT-PCR produces multiple copies of the cDNA with help of specific primer pair flanking the sequence to be amplified. PCR consists of repeated cycles of denaturation, primer annealing, and primer extension by DNA polymerase to amplify gene of interest exponentially. In a traditional end-point PCR, amplicons are run on agarose gel for detection with the help of primarily used fluorescent dye like ethidium bromide. An advancement of PCR is quantitative real-time PCR (RT-qPCR). It operates in the

same manner as that of the end-point PCR except that the amplicons are monitored in "real time" with fluorescent probes like SYBR green or TaqMan. SYBR green intercalates between double-stranded DNA strands that are generated as PCR products. Cycles advance, giving a fluorescent signal proportional to the amplification. Typically the target template DNA quantification is done by plotting an amplification curve with cycles number vs fluorescence. Cycle threshold (C_t) method, where a C_t value is reached when the fluorescence rises above the background level, is used for comparative analysis of RT-qPCR data. At the beginning of the assay, if more copies of target sequence are present, lesser C_t value is required to reach the level of detection. The fold change in the target sequence is expressed relative to the co-amplified housekeeping gene (Bustin 2005).

- *FACS (Fluorescence-Assisted Cell Sorter)*

The National Cancer Institute, USA, defines FACS as "A method of measuring the number of cells in a sample, the percentage of live cells in a sample, and certain characteristics of cells, such as size, shape, and the presence of tumor markers on the cell surface. The cells are stained with a light-sensitive dye, placed in a fluid, and passed in a stream by appropriate methods before a laser or other type of light. The measurements are based on how the dye labelled on the cells responds to the light."

Principle The objective of FACS is to count and sort/separate cells of interest from a mixture of cells based on various physical and molecular characteristics with the help of fluorescence. FACS machinery consists of three different systems: fluidics, optics, and electronics.

Methodology FACS is performed in three steps: sample labelling, flow cytometry, and detection and analysis (Fig. 10.2).

1. *Sample labelling:* This is a sample preparation step which includes labelling of cells or DNA/protein/surface marker on cells usually by incubation with a fluorochrome or fluorescent-tagged antibody specific for that particular moiety. It involves incubation of cells with fluorochrome-tagged antibody against protein of interest or dye. Cells are fixed if needed.
2. *Flow cytometry:* Labelled cells are then allowed to pass via the flow cytometer where they are passed and aligned through the flow cell in a single cell pattern with the help of hydrodynamic focusing obtained by applying differential pressure between sample and diluent (sheath/saline fluid). Each cell passing through the FACS nozzle is exposed to a laser which acts as an excitation light for the tagged fluorochrome or dye. The size, granularity, and amount of fluorochrome-tagged antibody on a single cell as well as the number of cells expressing that protein are determined by collecting the scattered light FSC and SSC (forward

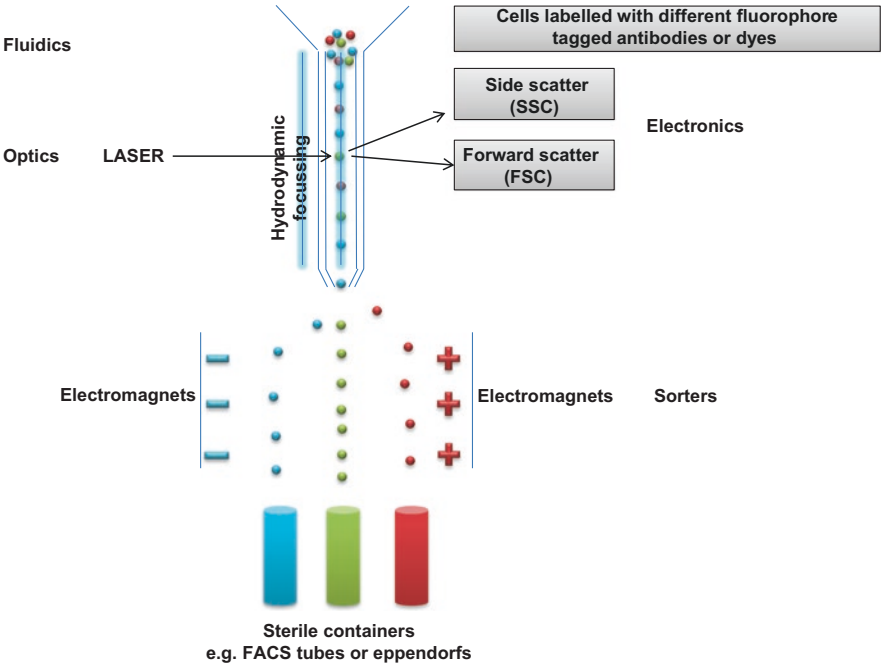

Fig. 10.2 Diagrammatic representation of basic flow cytometry

and side scatter) and emitted light from the fluorescently tagged antibody or dye. Sorting is performed on basis of charge with the help of electromagnets.

3. *Detection:* Light scattered and emitted is collected by the detector systems present inside the flow cytometer itself. PTMs are used for detection of the scattered and emitted light.

Applications It can be used for counting of cells, measuring size and granularity of cells, intracellular molecular staining (DNA or protein), surface biomarker staining (fluorescent antibody), and sorting the cells of interest from a mixture of cells.

1. *Detection of cancer cells/cancer stem cells/cells expressing particular proteins:* Often cancer cells exhibit different physical and molecular signatures as compared to normal cells. To identify and detect these potential biomarkers, FACS is regularly employed in research as well as clinical settings. For example, to identify cancer stem cells in cancer cell line or from tumor, cells are labelled with CSC markers such as CD44, CD24, CD133, etc., and cells can be sorted, separated, and cultured as a pure population.
2. *Apoptosis:* Chemotherapy is the first line of treatment in many cancer scenarios which employs various drugs for killing of cancer cells specifically. These drugs

cause apoptosis (programmed cell death) and/or necrosis in cancer cells. Induction of apoptosis is marked by the flipping of annexin onto the extracellular membrane surface of cells undergoing these phenomena. This particular event is exploited by FACS for detection of cell death. Cells are treated with compound/drug of interest in various concentrations. Cells are then labelled with fluorophore-tagged annexin antibody, e.g., annexin-FITC/annexin-PE, which specifically binds only to cells in which apoptosis is being exhibited by incubation. Another counterstain which can enter only through cells having pores due to necrosis induction, e.g., propidium iodide, is added to differentiate between necrotic and apoptotic population. These cells are analyzed via FACS for measuring population of cells undergoing apoptosis and necrosis, thereby giving a measurement of percent cell cytotoxicity, induced due to that compound/drug of interest at different concentrations, thereby also giving a measure of drug/compound efficacy (Adan et al. 2017; Orfao et al. 1995).

- *Enzyme assays*

Oncogene-driven changes in cancer cell metabolism like enhanced glucose uptake, increased lactate production, and reduced oxidative phosphorylation are advantageous to the growth and survival of tumor cells. Activities of various enzymes involved in cellular metabolism are altered in cancer cells. Enzyme assays give an idea about differential regulation of enzymatic activities in serum, tumor cells, or tissue lysates which can serve as biomarker or help in designing therapeutic strategies in cancer treatment. Principally all enzyme assays measure either consumption of the substrate or the formation of product over time. Constant readout of the enzymatic activity is recorded in continuous enzyme assays, while in discontinuous assays, reaction is stopped, and then final readout is noted. In both the cases, the important rule is substrate or product should not overlap with respect to the feature used for measurement. For example, LDH (lactate dehydrogenase) activity is a type of continuous assay. LDH is a glycolytic enzyme and reversibly converts pyruvate to lactate, thus facilitating anaerobic glycolysis in cancer cells. This reaction is coupled with oxidation of NADH to NAD+. As NAD+ is formed during the course of the reaction, absorbance of NADH at 340 nm (A_{340}) decreases which can be measured by spectrophotometer at specific time intervals. Subsequently, average absorbance difference per min (ΔA/min) is calculated which reflects activity of LDH in the sample. Commercialized glucose estimation kits utilize coupled enzyme assay analysis. The reagent in the kit consists of GOD (glucose oxidase), POD (peroxidase), and 4-AP (4-aminoohenazone). GOD converts glucose in the test sample (serum or culture medium) to gluconic acid with the generation of hydrogen peroxide. It further reacts with 4-AP in presence of POD to form a chromogenic product which is detected by spectrophotometer at 505 nm (A_{505}). The concentration of glucose in test sample is calculated by comparing with the absorbance of the standard glucose solution (Manyhart et al. 2016; Bisswanger 2014).

- *Techniques to Identify DNA-Protein Interactions*

Proteins bind to the specific sequence motifs on DNA and regulate cellular processes like transcriptional regulation and DNA replication and repair. DNA-transcription factor interaction is widely studied. Oncogenes can act as a transcription factors and also regulate cellular glucose metabolism.

– *ChIP (Chromatin Immunoprecipitation) Assay*

This method allows detection of protein-DNA interaction in living cells. The steps in ChIP assay are as follows (Fig. 10.3).

1. *Cross-linking:* In this step proteins interacting with the specific stretches of DNA like transcription factors are locked in DNA-protein complexes. This is achieved by treating biological samples with cell-penetrating cross-linkers like formaldehyde. The unreacted formaldehyde is quenched by glycine. Further cells are washed and can be stored at −80 °C till further use.
2. *Cell lysis:* Cells are lysed with the help of detergent like SDS and sonicated to shear cross-linked DNA into 200–1000 base pair fragments.
3. *Immunoprecipitation:* Sonicated cell lysate is diluted in a suitable buffer; antibody against the protein of interest is added and allowed to bind for overnight with rotation. Subsequently, protein G agarose beads are added to precipitate antibody which has now bound with cross-linked protein-DNA complex. Series of washing steps are performed to get rid of nonspecific DNA-protein complexes interacting with beads.

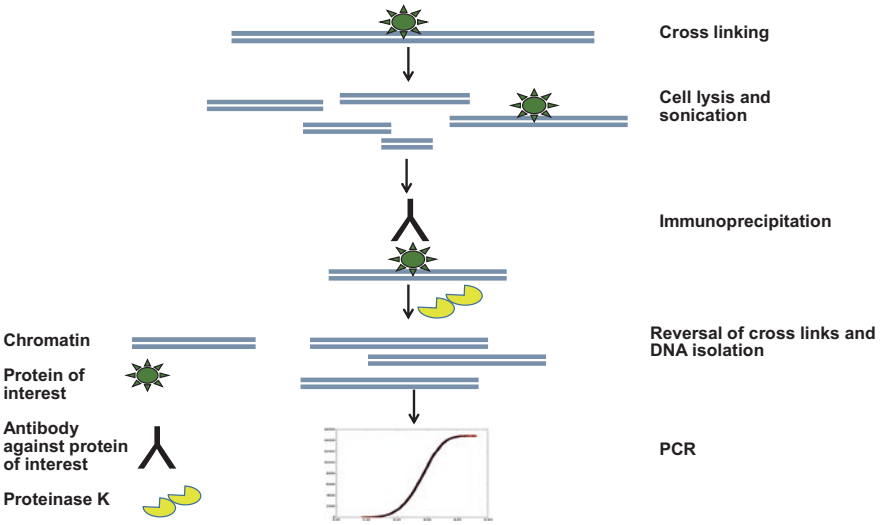

Fig. 10.3 Diagrammatic representation of chromatin immunoprecipitation (ChIP) assay protocol

4. *Reversal of cross-link:* To quantify target DNA or specific DNA sequence interacting with the protein of interest, cross-links between DNA and protein need to be reversed. This is achieved by extensive heating of immunocomplexes at 65 °C and proteinase K-mediated digestion of proteins.
5. *DNA quantitation:* DNA is isolated by phenol/chloroform method and precipitated by ethanol. By sequencing the DNA stretches interacted with the protein of interest, promoter regions for different transcription factors can be identified. Quantification of DNA-protein interaction can be achieved by coupling ChIP with qPCR (Nelson 2006).

- *EMSA (Electrophoretic Mobility Shift Assay)*

It is the widely used technique to detect protein-nucleic acid interactions. It is also known as gel retardation assay or gel shift assay. In the classical assay, nucleic acid probes are labelled with radioisotopes. On non-denaturing gel electrophoresis either polyacrylamide gel electrophoresis (PAGE) or agarose, labelled-nucleic acid probes migrate toward anode. Upon binding to interacting protein, mobility of the complex is retarded, and it migrates slower as compared to free nucleic acid probe. Protein-^{32}P labelled nucleic acid complexes are detected by autoradiography. Other than ^{32}P labelling, nucleic acid probes can also be tagged with biotin or fluorochromes. It is a simple, sensitive, and robust technique and requires minimum instrumentation as compared to ChIP. Binding site mutational studies are possible with this technique. The major drawback of the technique is the short half-life of ^{32}P. Another shortcoming is that protein-nucleic acid interactions can be analyzed in cellular lysates and not in living cells. To confirm protein identity in the complex, supershift assay with antibody specific to the protein of interest is utilized (Li 2004) (Fig. 10.4).

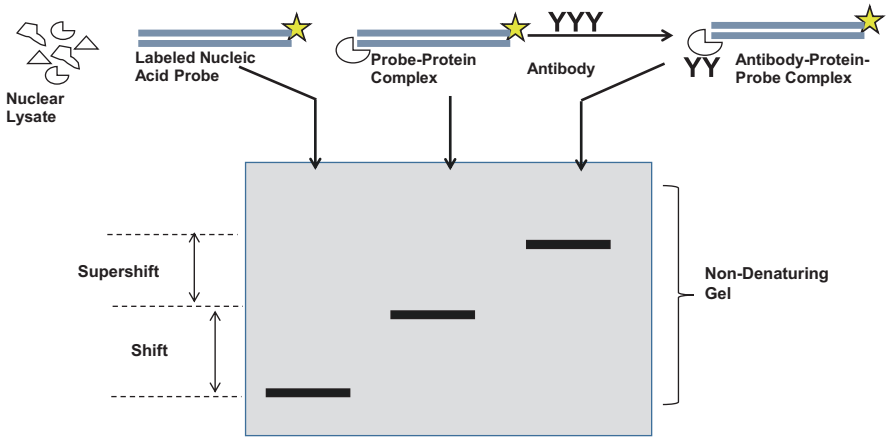

Fig. 10.4 Diagrammatic representation of electrophoretic mobility shift assay (EMSA) protocol

10.3 Applications in Cancer Therapeutics and Diagnostics

Molecular and cellular biology has contributed immensely in cancer therapy and diagnostics. The characteristics and advantages exhibited by cancer cells are a consequence of dysregulation in signaling of their dysregulated signaling. Unravelling these altered signaling pathways provides a plethora of potential targets for cancer treatment. Comparing cancer tissues with their normal counterparts of the same tissue by above mentioned genomic and proteomic techniques such as PCR, immunoblotting, sequencing, etc. has let to the discovery and targeting of the pathways which are responsible for immortal and proliferative phenotype of cancer cells.

Dysregulated signaling exhibited by cancer cells has often been exploited in pharmaceutics for diagnostic and therapeutic purposes. Since altered signaling causes overexpression or underexpression of genes, a variety of molecular biology techniques involving the diagnosis of these potential "biomarker" genes and normalizing their dysregulated expression is performed via various methods for detection and treatment of cancer. For example, in many cancers, mutations occur in genes controlling cell cycle which is the regulatory mechanism for proliferation of any cell. Alterations in these genes are the so-called cause of uncontrolled proliferation or cancer itself. Such genes which promote proliferation and are often overexpressed (gain of function) in cancer are called as oncogenes, e.g., BRCA1 and BRCA2, and those which keep the cell cycle-regulated proliferation in check and are underexpressed (loss of function) in cancer are called as tumor suppressor genes, e.g., p53. Dysregulated expression of oncogenes and tumor suppressors can be modified in order to treat cancer. Various gene editing techniques have been implied for this purpose. Along with gene editing tools, the discovery of monoclonal antibodies via the hybridoma technique is often used especially in breast cancer.

- *Application in Therapeutics*

1. *Gene editing tools:* Gene editing tools edit the genome in order to normalize the altered signaling exhibited by cancer cells and thereby stop proliferation. A number of gene editing tools have been implemented in the past, namely, TALENs, RNAi, CRISPR-CAS, etc.
   - *TALENs and ZNFs*: TALENs (transcription activator-like effector nucleases) and ZNFs (zinc finger nucleases) are chimeric nucleases that cause DNA double-stranded breaks, thereby editing the genome in order to silence the targeted oncogene permanently.
   - *CRISPR-CAS:* Clustered regularly interspaced short palindromic repeats are bacterial defense mechanism for destruction of foreign DNA, and Cas is an endonuclease which causes double-stranded breaks. This technique too involves silencing of the gene for achieving cancer treatment. The simplicity and specificity involved in this technique are unparalleled in the history of gene editing, a lot of research recently has been in progress with respect to CRISPR-CAS, and it definitely promises a bright future in cancer therapy. A

PD-1 CRISPR-CAS model is currently in clinical trials (Imran et al. 2017; Zhan et al. 2018).

2. *Monoclonal antibodies:* The invent of hybridoma technique in the past century has led to discovery of a new line of treatment altogether which essentially targets a particular type of cancer exhibiting specific biomarker overexpression. The overexpression of genes such as Herceptin in breast cancer and BCR-ABL in leukemia has effectively led to production and clinical application of trastuzumab monoclonal antibody which targets the HER2 receptor. Since these genes are specifically overexpressed in cancer cells, monoclonal antibodies provide a mode of targeted cancer therapy. Similarly various other antibodies have been discovered which target different proteins specifically overexpressed in cancer cells only. A list of FDA-approved monoclonal antibodies specifically in cancer therapy is described below.
   Rituximab (Rituxan®) targeting CD20 in B-cell lymphoma
   Trastuzumab (Herceptin®) targeting HER2/neu in breast cancer
   Gemtuzumab (Mylotarg®) targeting CD33 in acute myeloid leukemia
   Alemtuzumab (MabCampath®) targeting CD52 in chronic lymphatic leukemia
   90Y-ibritumomab (Zevalin®) targeting CD20 in B-cell lymphoma
   131I-tositumomab (Bexxar®) targeting CD20 in B-cell lymphoma
   Bevacizumab (Avastin®) targeting VEGFR colorectal cancer
   Cetuximab (Erbitux®) targeting EGFR colorectal cancer (Stern and Herrmann 2005; Scott et al. 2012)

- *Application in Diagnostics*

1. *PET (positron emission topography) scanning:* Cancer cells follow an altered glucose metabolism pathway as explained by German scientist and Nobel Prize winner Otto Warburg, and they tend to uptake and utilize more glucose than other cells. This dysregulated but fast rate of glucose consumption which takes place via a series of dysregulated signaling of molecular and metabolic alterations is exploited for the diagnosis of cancer by PET scanning. In PET scanning, radioactive glucose/18F-fluorodeoxyglucose (FDG) is injected in patient body which can be detected externally or noninvasively. The tissues which take up labelled glucose can be visualized in the order of color coding as a function of their glucose consumption, e.g., a tissue which consumes less glucose is visualized in a different color and the tissue which consumes more glucose is displayed in a different color. Since PET scan shows tissues where more of the abovementioned FDG is utilized, cancer cells/tumors owing to their very high rate of glucose consumption are easily detected in a PET scan (usually a tumor shows bright/differently colored area in a PET scan). This method has unparalleled application as a technique in cancer biology as it is used not just for the detection of cancer but also for checking the effectivity of a chemotherapeutic drug by monitoring tumor reduction and metastasis and also in anti-cancer drug discovery (Gambhir 2002; West et al. 2004).

2. *Biomarkers:* Cancer biology is a constantly growing research ground. Decades of research has contributed toward the discovery of a number of biomarkers which can be quantitated by a variety of techniques such as mass spectrometry, immunohistochemistry, immunofluorescence, etc. These biomarkers can be very crucial in determining the type of treatment method in cancer patients. Cancer biomarkers can be in the form of proteins expressed intracellularly or on the surface of cancer cells. FACS can be used to detect these specific markers to distinguish and identify the presence or absence of cancer cells in a patient especially those present on the surface. Moreover, resistant cancer cells and metastatic cancer cells can be detected by FACS. For intracellular biomarkers, techniques such as FISH, IHC, and mass spectrometry can be used along with FACS (Tothill et al. 2015; Kumar et al. 2006).

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Analysing Cancer Signalling Pathways: A Structural Bioinformatics Approach

11

Jitesh Doshi, Shubhankar Dutta, and Kakoli Bose

Abstract

Regulation of cancer and its development is a complex process that combines an aggregation of numerous mutations with dynamic changes and a complicated cross-talk among various types of cells involved in tumours. Poor understanding of the underlying cellular mechanisms involving various biological molecules associated with cancer has resulted in confusing prognosis. Thus, interpretation of relationships between these biological molecules, networks and pathways is necessary to understand the intricacies of cancer biology. Emergence of a specialised branch of biology, namely, bioinformatics, has enabled cancer biologists to bridge the gap between genomics and proteomics. One of its subdomains is structural bioinformatics, which includes techniques such as molecular modelling, high-throughput docking, mutation analysis, network modelling and drug designing. Robustness of the algorithms and pipelines involving these techniques has made efficient handling of heterogeneous and ever-evolving tumour data possible. In this chapter, we have tried to elucidate the application of structural bioinformatics in the analysis of cancer signalling pathways.

Keywords

Structural bioinformatics · Cancer · Protein structure · Protein-protein interactions · Interactome · Mutation · Network modelling · Network analysis · Signalling pathways · Cancer signalling · Tumour biology · Pathway visualisation

The authors Jitesh Doshi and Shubhankar Dutta have contributed equally to this work.

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11.1 Introduction

Cancer generally advances with genetic and epigenetic alterations involving signalling pathways of cell cycle progression, cell growth and apoptosis (Sanchez-Vega et al. 2018). This assists cells in escaping the homeostatic control, thus allowing them to proliferate with aberrations (Sever and Brugge 2015). These genetic alterations manifest into deviations in characteristic structures of proteins involved in related cellular processes. In turn, the changes happening in the three-dimensional (3D) structures of protein molecules result in the state of irregularity in the cell. Protein structures have known to be a very important part of cellular machineries as they provide a functional importance to the proteins and carry out desired task. While the protein structure is very important in understanding the molecular processes that define life, there are several challenges in determining it at various stages. These challenges arise due to many factors including nature of the protein, cost and time involved as well as high rate of failure in carrying out these studies. Ultimately, it slows down or even sometimes eliminates structural studies (Slabinski et al. 2007). This has created a large gap between the molecular structural data and enormous amount of genomic and sequence data available from high-throughput experiments. Efforts are being taken globally via high-throughput structural genomics centres to bridge this gap. In an effort to solve more and more structures and close this gap, not only the important cellular mechanisms have been unravelled, but also important molecular structures have been mapped to a particular process along with their interactions with entities like inhibitors, cofactors, substrates, etc. (Slabinski et al. 2007), thus opening a new avenue of structure-based drug discovery. With the advent of advanced computer technology emerged a new field of bioinformatics, which utilises the tools and techniques from computer science to solve problems in biology and medicine. Structural bioinformatics is a subdomain within the evolving field of bioinformatics, which exists to fulfil two major goals, viz. inventing new methods to deal with the molecular structures (structure visualisation, determination/prediction) and using these methods to create new information (structure analysis) (Bourne and Weissig 2003). Bioinformatics depends on large-scale complex data, which cannot be handled manually. Field of structural bioinformatics or computational structural biology started with emergence of database such as Protein Data Bank or PDB (https://www.rcsb.org/) (Berman et al. 2000), which deposits 3D structures of biomolecules including protein, DNA and RNA in computationally understandable form. It is now an established fact that biology has largely transformed into a data-driven, quantitative science and large amount of high-throughput data available from massive experiments is driving today's research. Availability of such a vast amount of data has opened several possibilities and helped us zoom into life in greater details. Genome-wide network of interacting partners is now available, and it provides us with systems-level understanding of biological entities. These networks, referred to as *interactomes*, are playing a crucial role in understanding the life processes, and thus, description of biological networks is necessary to understand these complex processes and diseases arising from their alterations (Petrey and Honig 2014). These networks are still not entirely

solved due to lack of structural data, and there remains a gap between proposed function and mechanism by which these events occur. It also cannot shed light on mechanism by which a particular disease is propagated or what changes occur in the mechanism (Petrey and Honig 2014). Fundamental requirement for initiating such studies is availability of a description of 3D structure, and structural bioinformatics starts right there. Integration of 3D structure information with pathway data defines the field of structural genomics, which gives a better understanding of biological phenomena at systems level. Advancements in structural bioinformatics have provided tools to predict protein structures and also to correlate functional likenesses to structural similarity. These tools will help bridge the gap between sequence and structure data and allow us to utilise this structural information without physically having to determine the structure using conventional methods such as X-ray crystallography or nuclear magnetic resonance (NMR). Having said this, it is important to explore the recent advancements in the field of structural bioinformatics and its allied branches, their possible applications in improving our understanding of the signalling pathways and their limitations as well.

This chapter thus attempts to investigate current state of structural bioinformatics field and review commonly used methods and their applications in understanding cancer signalling pathways.

11.2 Protein Structure Prediction

Despite knowing that the protein structure is important to understand the mechanism of cellular functions, dearth of available structure data pose a lot of challenges in studying these processes and also in developing novel methods to determine the structure. The last two decades have seen a great deal of structures being deposited in Protein Data Bank (PDB) (Fig. 11.1). Partially, this has been possible due to high-throughput structural genomics centres being established worldwide. Growth in data and easy accessibility of powerful computer systems as well as algorithms have simultaneously provided a driving force to the alternative theoretical methods of structure determination (Fig. 11.1).

Classically, three different approaches have been used by all researchers to predict the 3D structure of protein of their interest. All available services for protein structure prediction can be classified into the following categories: homology modelling, fold recognition and ab initio. The volume of available information determines which category to choose.

11.2.1 Homology Modelling

Homology modelling is one of the simplest and most commonly used techniques for computational prediction of a protein structure. It is based on two main assumptions (Bourne and Weissig 2003):

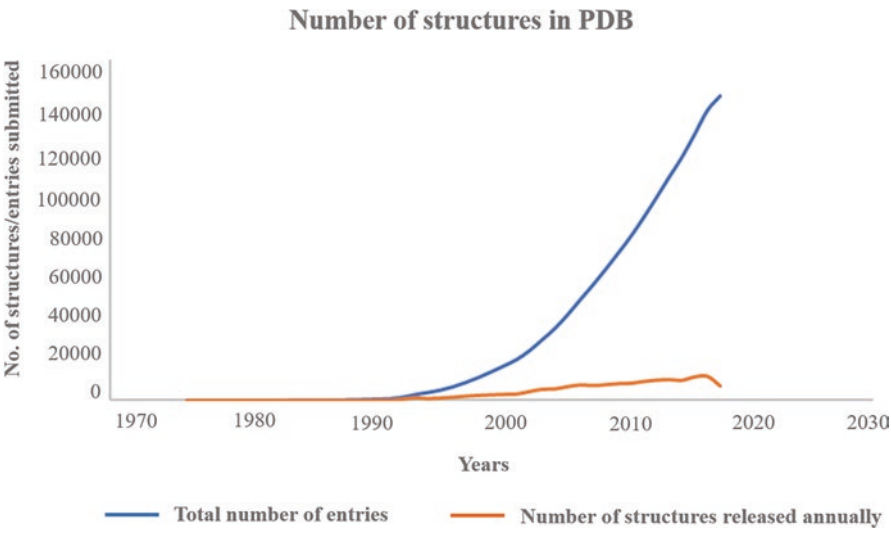

Fig. 11.1 Growth of solved structures in PDB. Increase in number of biomolecular structures deposited in Protein Data Bank (PDB) over the last two decades is huge, which can be attributed to advances in structural biology

1. Particular amino acid sequence is responsible for imparting a characteristic structure to the protein.
2. During evolution, the structure (that is responsible for function) is more conserved than the sequence. Therefore, two similar sequences are assumed to possess similar structures.

Homology modelling essentially requires that the structure of a homologous protein (template) be already available in PDB. Homology modelling algorithm then aligns the two proteins and copies the structure of a template protein to the protein of our interest pertaining to the above-mentioned assumptions. Various web-based and standalone software services are available today for free and easy setting up of homology modelling. Commonly used tools are Modeller (standalone, python-based tool for comparative modelling using spatial restraints) (Eswar et al. 2006), Swiss-model (fully automated user-friendly web-server for fast modelling of protein structure) (Arnold et al. 2006), I-TASSER (automated, web-based tool for protein structure and function prediction) (Yang et al. 2015), etc. These tools are freely available to scientific community and provide easier ways to build protein structures from homologous template structures. There is a plethora of tools available today for building protein structure models, and it becomes increasingly confusing to choose one best tool. Along with these freely available tools, several commercial packages are available in the market that helps build faster, easier and better-quality models. To assess the quality of numerous algorithms available, CASP or the Critical Assessment of Structure Prediction has been established. CASP is a community-wide global experiment for protein structure prediction. It helps in

deciding the status and focus of the current efforts in structure prediction realm and assists in determining the future direction (Moult et al. 1995). This experiment is carried out every 2 years since 1994. Results of the most recent CASP12 (2016) elaborate on the assessment of methodologies submitted by individual groups as well as online servers (Moult et al. 2018). At any point in time, it is a good idea to visit CASP website (http://www.predictioncenter.org) to gain most recent updates in structure prediction. Apart from predicting the structure of an unknown homologous protein, homology modelling is very useful in case of cancer-related studies. Mutations in many proteins involved in signal transduction are hallmarks of cancer. To study the effect of these mutations, homology modelling serves as a powerful tool by allowing a wild-type protein to be a template of its mutant variants from various cancer tissue samples, thus permitting us to investigate the mechanism of action of that particular protein and its probable effect on the cancer signalling pathway. This essentially paves way to understand structure-function relationships. A study demonstrates an elegant use of homology modelling to predict the functional effect of single nucleotide polymorphisms (SNPs) of HOXB13 (Homeobox B13) and investigate the role of these mutations in hereditary prostate cancer (Chandrasekaran et al. 2017). In this study, a complete HOXB13 protein structure was created using homology modelling tool – Modeller v9.17. Then, a set of studied SNPs were mapped to this structure using mutation tool in Swiss PDBViewer software to create mutant structures. All the structures were further subjected to energy minimisation to study the effect of these mutations on structure of HOXB13. Although the experiment needs validation using complementary wet lab studies, it does give us a lead on the SNPs that might cause damaging effects to the structure. SNPs causing increased as well as decreased stability to HOXB13 structure were also identified by analysing the total energy of the mutants.

Homology modelling is still limited by unavailability of homologous protein structures, development of algorithms for complex proteins, less accuracy and precision when applied to certain class of proteins like membrane bound proteins, intrinsically disordered proteins, etc. These limitations can be circumvented by sincere efforts of structural genomic centres to solve more and more structures. Moreover, by efficient blending of machine learning and artificial intelligence in structure prediction methodologies, along with incorporation of experimental data such as mass spectrometry, the existing vacuity at the interface of structural and cell biology research in cancer can be erased.

11.2.2 Template-Free Modelling

Though homology modelling is a widely used technique to determine protein structure computationally, a suitable template structure is not always available for several novel proteins of interest. In such scenarios, other two techniques of theoretical structure determination are available for use, viz. fold recognition (aka threading or fragment-based modelling) and ab initio (aka de novo) modelling, collectively known as template-free modelling techniques. From several studies, it has been

hypothesised that a large number of different protein sequences can form only a limited number of protein structural folds or motifs. This might be attributed to various stereochemical constraints (Wang 1996) or frequencies of different events during evolution (Mushegian 2007). Assuming this principle to be true, fold recognition methods work by using folds similar in sequence to the small fragments of protein of interest as templates and then iteratively build the complete structure. For remaining proteins that do not have statistically significant similar sequences, ab initio class of methods are used. Ab initio modelling derives structural models using principle from physics-based methods (Vallat et al. 2015). Although these template-free methods are less reliable than the models derived through homology modelling, nevertheless they provide some crucial leads on the overall structural organisation of the proteins when validated using complementary experimental biophysical studies involving tools such as fluorescence spectroscopy and circular dichroism (Bejugam et al. 2013). Several tools, well-known for decades, are still used widely owing to their popularity such as Robetta and Phyre2. Both Robetta (automated tool for protein structure prediction using comparative or fragment based modelling) (Kim et al. 2004) and Phyre2 (provides prediction of protein structure, function, and mutations using advanced remote homology detection methods) (Kelley et al. 2015) are freely available as web services for academic and research users. I-TASSER is one of the popular servers available for ab initio modelling. Its popularity has only grown in the last few years. Many new algorithms are still being developed, but those either are not available for everyone yet or possess a layer of complexity that makes them difficult for new users. Similar to homology modelling methods, CASP also assesses template-free methods and ranks them based on various parameters. These rankings help determine the course of development of these methods and assist in choosing the best one. Although a lot of advancement has happened in the field, popular servers mentioned above always compete for the top rankings in CASP assessment.

The first step in studying any cancer signalling pathway using structural bioinformatics approach is to obtain the structure of protein molecules involved in that pathway. PDB is a primary resource for obtaining solved structures of these proteins as it is a repository of experimentally determined structures of biomolecules at the highest possible resolution. Unavailability of solved structures leads us to the methods of theoretical determination of protein structure discussed in Sect. 1.2. Once structure is available, a large array of analyses can be put in a pipeline to understand the pathway in greater details.

11.3 Protein-Protein Interactions

In a common abstract representation of interactome, proteins are represented as ‘nodes’, and interactions between them are represented as ‘edges’ of the graph. This provides a comprehensive view of a particular biological process that aids in understanding of molecular mechanisms of diseases including cancer, where disruption of signalling pathway or ‘network’ occurs (Kann 2007). To understand these

processes at molecular level and to relate their functional implications, structural details need to be incorporated into network. These details can be obtained from atom level description of proteins' 3D structures and their interaction interfaces (Kar et al. 2009). These interactions are the basis of most of the cellular processes, and they occur due to attraction between specific binding sites on protein surfaces. Identification of binding partners is a straightforward way to predict the function of a protein. It requires known function of at least one of the binding partners, which facilitates functional prediction and pathway assignment of other proteins in the complex (Keskin et al. 2008). Thus, identification of protein-protein interactions (PPIs) is helpful to understand the process better, to elucidate dynamic regulation of these interactions in a pathway and to exert functional significance to new discoveries. Prediction of PPI is also important in the process of computer-aided drug discovery at target selection stage. There are several types of proteins identified in a biological network and categorised as hubs or bottlenecks depending upon degree of distribution and betweenness (Kar et al. 2009). Their characteristic features, unique distribution and role in cancer make them a worthy target and an essential entity in study of cancer signalling pathways. Various methods of predicting PPIs computationally are in use today. Some of the common principles these methods rely on are evolutionary relationships, co-expression studies, co-localisation, genome- and sequence-based methods, etc. (Bourne and Weissig 2003). At structural level, identification of interacting partners of a network is a more challenging task yet a more accurate measurement of interaction. Protein-protein interactions, especially among signalling pathways, are gaining a huge importance as possible anti-cancer targets (Ivanov et al. 2013; Zhang et al. 2016; Huang et al. 2008). This is due to their direct involvement in the disruption/regulation of the signalling pathway, thus providing better understanding of interactions that are important to the development and treatment of disease.

11.3.1 Molecular Docking

A class of methods called 'molecular docking' is a well-established domain within structural bioinformatics to model the 3D structure of a complex of two interacting partners. Docking programs essentially compare the complementarities of two structures and predict whether the partners under consideration have significant potential to interact with each other. Molecular interactions at cellular level are driven by energetic favourability and stereo-chemical complementarities between the partners. Docking programs also make use of similar energy functions to evaluate various possible orientations of the two participating molecules and rank them from lowest to highest energy scores calculated by theoretical approaches. Docking programs can be further classified into protein-protein docking, protein-ligand (small molecule) docking or protein-nucleic acid docking, depending on the nature of binding partners. Pertaining to our discussion of investigating cancer signalling pathways, protein-protein docking is an appropriate tool to focus on. Docking protocols started emerging in early 1970s, even though substantial computing power

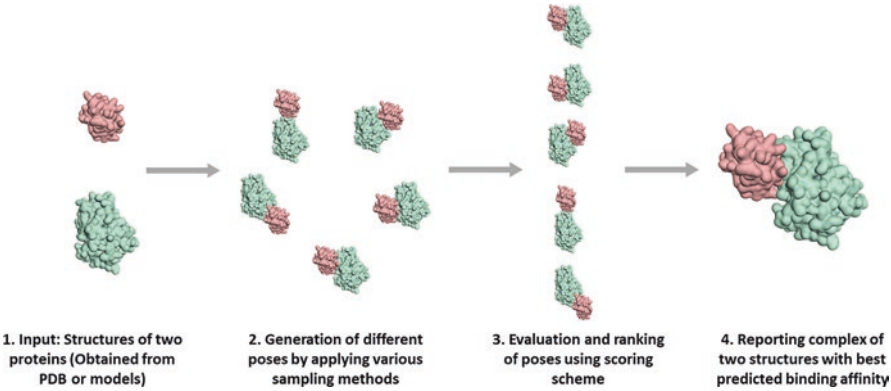

Fig. 11.2 Steps involved in docking of two molecules. General procedure for docking algorithms involves generating various poses to create large number of binding interfaces between two given structures (1). For rigid body docking algorithms, these poses are based on only six degrees of freedom – three translational and three rotational. Flexible docking generates a large number of poses in the conformational space (2). Evaluation is another stage where various docking algorithms differ from each other. Based on information used in calculations, these scoring functions could be either force fields based, empirical in nature, knowledge-based or machine learning based (3)

was not available. Early studies reported computational theoretical models for well-studied proteins like haemoglobin (Levinthal et al. 1975) and chymotrypsin (Platzer et al. 1972). These methods have grown significantly since then and play a very important role in routine structural studies. A variety of approaches has been used over the years to solve the problem of modelling interactions between two structures. Most of the widely used docking protocols use either geometric complementarity between the two partners or steric complementarity between the binding interfaces.

Docking problem can be described as follows: given the structure of two proteins, find a 3D structure of their complex as close to what it would appear in real life. Before understanding how solution to this problem can be applied to a signalling network, it is important to understand various types of docking simulations and challenges it poses at various stages. Nature of molecular interactions entails a significant amount of conformational changes in the 3D structure of protein upon interaction. Based on whether a docking program considers these conformational changes into calculations or not, they can be classified as flexible or rigid docking. Unlike rigid docking, flexible docking allows a larger degree of freedom to cover more conformational search space, thus providing more accurate estimation of the complex (Vakser 2014). All docking programs essentially work by sampling out large number of possible spatial configurations of given two structures and ultimately evaluating them on the basis of various scoring schemes to approximate the binding affinity between them (Fig. 11.2).

There are a large number of docking algorithms available, and it is quite difficult to pinpoint one particular algorithm. Similar to CASP experiment for protein

structure prediction methods, CAPRI or Critical Assessment of PRediction of Interactions is a large-scale, community-wide experiment, which provides benchmark datasets for testing protein-protein interaction prediction programs or servers. Most of the widely used programs make use of this platform to establish the quality of their software or to improve upon earlier versions. Protein-protein docking problems are computationally very expensive and require access to the high-performance computing facility to run the calculation smoothly. Various docking servers provide free access to such facility and allow one to use their program for docking studies. For someone who is new to the field of structural bioinformatics, it is a good idea to use these servers as starting point of their work. ClusPro (https://cluspro.bu.edu/), which is a 3.0 version now, is a popular choice for most protein-protein docking studies. Although there are many other algorithms that may be comparable to ClusPro in terms of results, ClusPro remains an obvious choice due to its user-friendly, easy to use interface and level of control over calculation (advanced mode) as well as results. ClusPro uses rigid body sampling, clustering and energy minimisation in order to provide good results (Kozakov et al. 2017). Interaction energy calculated by ClusPro follows the equation:

$$E = w_1 E_{rep} + w_2 E_{attr} + w_3 E_{elec} + w_4 E_{DARS}$$

where E_{rep} and E_{attr} are repulsive and attractive contributions respectively, E_{elec} is an electrostatic term, and E_{DARS} is a structure-based potential constructed by DARS or the Decoys as the Reference State method to account for the free energy change due to removal of water molecules from the interface. w_1, w_2, w_3 and w_4 are the weights for individual energy terms that are optimised for specific types of problems. Several advanced options to incorporate more information in the calculation, easy job submission and results with multiple formats to use as per the case make ClusPro a good place to start performing protein-protein docking studies to understand PPIs. ZDOCKpro is a novel protein-protein docking algorithm, currently available as a part of commercial Accelrys Discovery Studio© suite. ZDOCKpro works in two stages. In the first stage, ZDOCK performs rigid body docking with pairwise shape complementarity, and in the second, RDOCK performs force field-based refinements of the docked poses, improving the quality of rigid body-based models of the interaction (Pierce et al. 2014). ZDOCKpro has consistently performed well in CAPRI experiments over the years. ZDOCK (http://zdock.umassmed.edu/) is also available as a free, web-based server to perform docking calculations, but it does not provide RDOCK module to perform refinement of the docked structures. SwarmDock (https://bmm.crick.ac.uk/~svc-bmm-swarmdock/) is another server with potential, and it has been validated with CAPRI benchmarks. SwarmDock is a flexible docking algorithm and provides an automated pipeline that include repairing structure, clustering and reporting the best poses (Torchala et al. 2013). Depending on the size of the system and number of people currently using these services, it takes a few hours to few days to get the results of protein-protein docking. There are several other resources to perform protein-protein docking, free as well as commercial, which can be used on case-by-case basis depending on the feasibility, nature of protein, quality of protein structure, time at hand, etc. In a

nutshell, if 3D structures of two proteins are available, running a protein-protein docking algorithm on these structures would not only provide a possibility of the interaction but also will highlight the amino acid residues taking part in the interaction. This information then can be used to investigate the role of the interaction in a signalling pathway.

All the methods discussed above provide a simple measure to study a pair of proteins. In order to study the interactome or at least a specific signalling pathway, a large-scale, high-throughput docking experiment needs to be carried out. Limitation on structure availability is one of the challenges in these studies. This problem can partially be circumvented by modelling the proteins as templates are available for most of the interacting proteins of the genome. However, improvement of the accuracy of high-throughput methods is still needed for obtaining more precise structural bioinformatics data (Vakser 2014).

11.4 Mutation Analysis

Simultaneous analysis of protein sequence and structure is necessary to extract the functional or phenotypic effects of various genetic variants or polymorphism. Most of the current and upcoming projects are largely focused on the generation of a catalogue of single nucleotide polymorphisms (SNPs) occurring in human population, locating their genomic position, annotating them and finding out their distribution pattern (Sachidanandam et al. 2001). Occurrence of SNPs varies from person to person, and this frequency study along with their location and annotation has been used to understand disease risk and severity. Mapping an SNP to identify whether it is segregating with a disease, or more common in affected versus unaffected, can determine the closeness of the SNP to the disease-causing mutation (Wang et al. 2011a). Analysis of any non-synonymous variant in the gene-coding region requires proper strategy to interpret and quantify its functional role. However, it is necessary to distinguish between functionally significant variations and likely neutral variants. This problem is also known as driver versus passenger problem where a passenger variant has no role in progression of a particular disease (mostly in the field of cancer) (Pon and Marra 2015). A large number of tools and databases have been developed to categorise these mutations where their functional impacts are studied either through evolutionary, sequence and structural information or through annotation derived from protein databases.

11.4.1 Mutation Repository and Databases

The most popular mutation repository where both manually and computationally curated SNPs are stored is dbSNP database (Smigielski 2000) at the NCBI. It is a very useful database where annotation of each SNP is given along with their frequency of occurrence in a control population. The largest resource for this database is the 1000 Genomes Project which feeds a large amount of SNP information to the

database (Altshuler et al. 2010). One of the striking features of the SNP information provided here is that each SNP is annotated with a minor allele frequency (MAF) value calculated from the 1000 Genomes Project where frequency higher than 0.5 represents common variant in the population (Tabangin et al. 2009). The main objective of mutation databases is to find out whether the identified SNP or variant is novel or not. Apart from dbSNP, there are some more human SNP databases like the Human Gene Mutation Database (HGMD) (Stenson et al. 2009), Allele Frequency Database (ALFRED) (Cheung et al. 2000), Frequency of Inherited Disorders Database (FINDbase) (van Baal et al. 2007), Protein Mutant Database (PMD) (Kawabata et al. 1999), Online Mendelian Inheritance in Man (OMIM) (Hamosh et al. 2000) and so forth. All these databases contain SNP-related information mostly derived from the literature. Specific gene locus mutation databases like the Breast Cancer Gene Database (BCGD) (Baasiri et al. 1999), p53 Gene Mutation Database (Béroud and Soussi 1998), Haemophilia Mutation database (HMD) (Giannelli et al. 1998), GRAP mutant Database (GPCR family proteins) (Isberg et al. 2016), LDLR Mutation Database (Varret et al. 1998), Long QT syndrome database (Modell and Lehmann 2006), etc. contain valuable information about disease-causing SNP and their role in multiple cell signalling pathways. Hence, before starting any mutation-related research, all must look into various SNP databases (full list can be found in human genome society website) (http://www.hgvs.org/).

11.4.2 Retrieving Functional Information from Primary Protein Sequences

Throughout the course of evolution, some regions in a protein sequence remain conserved. Residues in these regions can be important for protein structure or function, and it has been observed that disease-causing SNPs frequently occur at these positions. One of the most common methods is multiple sequence alignment (MSA) for identification of the pathogenicity of a mutation. Servers like ConFunc and ConSurf can be used for visualising and mapping of SNPs on protein structure using different colour format (Wass and Sternberg 2008). Apart from identifying the SNPs, it is also necessary to quantify the physicochemical properties of the mutated residues in terms of charge, hydrophobicity, hydrophilicity and size. These mutated residues are integral parts of mutated sequences, which can be globally aligned with the wild type and analysed using tools like MutationTaster (Schwarz et al. 2010), VarScan (Koboldt et al. 2009), etc. However, not only SNPs present in a stretch of motif can cause alteration in the protein function but can be detrimental to protein's activity beyond post-translational modification. At cellular level, mutation may also result in disruption of the localisation signal, hence deterring the correct subcellular location of the protein which is required for its functioning. Therefore, preliminary identification of these stretches of residues that are affected by mutations is necessary to study them further at structural and functional level.

11.4.3 Mutation Study at Structural Level and Their Impact on Protein Function

To study the role of any mutation, the mutation must be mapped on the protein structure. The protein of study may or may not have an experimentally determined structure available in Protein Data Bank (PDB). As discussed earlier, using homology and ab initio modelling, various protein 3D structures can be modelled for which experimentally determined (X-ray diffraction, nuclear magnetic resonance, etc.) crystal structures are not available. On these structures, mutations can be mapped to determine the location of the mutated residues in three-dimensional space as it is very important to understand whether the affected residue is in the core region or at the surface. Various previous studies have found that protein unfolding can be triggered by the destabilising effect of the mutations at core residues, whereas surface residue mutations can specifically affect the interface interactions and eventually alter the binding energies of protein-protein or protein-ligand complex (Sheinerman et al. 2000). Not only single-site mutation but alterations at conserved stretches of structural motifs may also disrupt the protein function by affecting its molecular mechanisms. Functioning of these kinds of motifs depends on the proper arrangement of their secondary structure, which may further facilitate both intra-residue and inter-residue interactions. In a protein structure, chances of residues to form α-helices, β-strands or loops depend on their specific inclinations towards the particular secondary structural elements. Residues like alanine, leucine and methionine tend to form helical conformations, whereas aromatic residues like tyrosine, tryptophan and phenylalanine prefer β-strands. On the other hand, glycine and proline are known as secondary structure breaker (Imai and Mitaku 2005). This difference in propensity of a particular residue to form a secondary structure may shed light on the properties of that residue and the effect it may bring due to any mutation. Moreover, in-depth study of alterations in the overall structural occupancy of mutated residues in 3D space would help us understand the individual as well as collective roles of the protein in protein-protein interaction (PPI) network.

11.4.4 Impact of Mutation on PPI Networks

An intercellular process very rarely gets mediated by a single protein; instead, a molecular machinery comprising multiple interacting proteins arbitrates the particular process. It is seen that disruption in the functioning of any one or more proteins due to various mutations can collapse the whole machinery, resulting in disease conditions. Thus, while studying PPI networks for any diseases like cancer, it is necessary to identify the possible disease-causing mutations present in the proteins involved. As discussed earlier, these mutations are mostly the missense class of mutations (non-synonymous), which are involved in amino acid substitutions, causing changes in protein sequences and structures. Till date, various automated pathway extraction methods have been developed using PPI network where these mutations are treated as either driver or passenger to the network, keeping in mind

that distinct amino acid sites within a protein can mediate different functions (Pon and Marra 2015). To solve this driver versus passenger problem, one must study data at genomic level. Nowadays, a multitude of mutations identified by genome sequencing are passenger mutations that have zero impact on the progression of cancer. On the other hand, there are few driver mutations which are found in various disease/patient samples that can cause selective growth of tumour cells at advanced stages (Kalari and Pfeifer 2010). Differentiating driver from passenger mutations is crucial towards lowering the number of false positives in the interaction-driven cancer networks. One of the most effective and popular approaches to distinguish between drivers and passengers are frequency-based methods that take background mutation rate (BMR) into consideration to compute the frequency of mutation for each gene or region (Wang et al. 2011b). In these methods, genes that are mutated at higher rates than expected are declared as cancer driver genes. However, it is very important to have a precise estimation of the BMR before applying for the frequency-based methods. So, various sophisticated tools like MutSigCV (Lawrence et al. 2013), InVEx (Gonzalez-Perez et al. 2013) and MuSiC (Dees et al. 2012) are used to estimate BMR across chromosomal locations and mutational spectra for a particular cancer sample.

Application of frequency-based methods may not be feasible when the studies are to be done at protein structure level. Hence, tools like SIFT (Sorting Intolerant from Tolerant) (Ng and Henikoff 2003) and PolyPhen-2 (Polymorphism Phenotyping v2) (Miosge et al. 2015) are used which can predict the mutation intensity on the basis of the functional impact of these variants on protein activity. These tools are considerably robust but not specialised in cancer gene prediction. Hence, some specialised annotating software like MutationAssessor (http://mutationassessor.org/r3/) and CHASM (Cancer-specific High-throughput Annotation of Somatic Mutations) (Carter et al. 2009) are combined along with SIFT and PolyPhen-2 to specifically assess the functional impact of variants in cancer. Apart from these, methods like TransFIC (TRANSformed Functional Impact for Cancer) (http://bbglab.irbbarcelona.org/transfic/help), which considers the basal tolerance to germline single nucleotide variants, and CONDEL (González-Pérez and López-Bigas 2011) are also used in various pipelines for studying the impact of mutations on proteins involved in various pathways and networks.

11.5 Network Modelling and Analysis

Analysis of networks is necessary to understand the functional relationships among gene expressions, activation of various transcription factors and cell signalling pathways (Barabási and Oltvai 2004). An array of network analysis and inference tools is present for the construction of network topologies (or interaction maps) surrounding transcriptional and proteomic measurements. Using them, any regulatory network applicable to oncogenic signalling can be developed that can provide a proven platform to decipher complex interactions/causalities in cancer biology.

11.5.1 Generating Network Topologies

Tools like Cytoscape (Shannon et al. 2003), BisoGenet (Martin et al. 2010), etc. build a structured network by taking PDB files as input and use different algorithms to identify the interactions of the protein at the residue level. Every algorithm is based on the statistically proven observation that if protein A activates protein B, then high levels of protein B are expected whenever level of activator A is high. The corollary to it is that statistical correlation between two or more proteins indicates potential interaction between them. Computationally, these statistical inputs can easily be introduced to verify a series of such hypotheses within seconds and provide a scoring system to the molecular interactors (proteins). A large number of statistical frameworks have been successfully applied to search for the most predictive modulator set by using the information of the modelled target's behaviour (Mitra et al. 2013). For example, structures in human T cells were reconstructed using the concentration of phospho-proteins in individual cells where the Bayesian concept of network topology was applied (Schäfer and Strimmer 2005). The same statistical approach correctly reconstructed yeast metabolic networks from gene expression data (Pe'er et al. 2001).

These kinds of data-driven approaches require hundreds of samples to gain the statistical resolution for the construction of molecular interaction topologies. So, another powerful strategy in network biology is to simplify models. Simplification of models is done by grouping proteins into modules based on their common regulatory mechanism that is also referred to as the 'module-network' approach (Bonnet et al. 2010). By pooling many similar proteins together, the module-network framework considerably escalates the statistical resolution to determine regulatory influences (Akavia et al. 2010).

11.5.2 Identifying Key Nodes in Cancer Network Models

Module-based network modelling helps in building template networks using statistical resolution methods where different genes or proteins are grouped into a module on the basis of their roles in the network. However, due to perturbations and other external factors, these modules may function arbitrarily, thus affecting the overall network model. Thus, it is necessary to identify the key components or nodes of the network. Furthermore, the connections among the nodes must be modelled on the basis of their responses toward external stimuli. This kind of framework is referred to as 'differential network' (Ideker and Krogan 2012).

Designing of a differential-network model is required to understand how the hub or the central component of the network is getting affected. Data-driven computational approaches like RNA sequencing, chip-seq sequencing, exome sequencing, etc. can be used to (a) detect the surplus components interacting with the affected nodes, (b) perform qualitative and quantitative analyses of the perturbed interactions and (c) build a model depicting the propagation of network perturbations through those surplus components for the generation of intended phenotypes like

response to the drug, cell proliferation, tumour cell invasion, etc. For example, there were cases where in the absence of external stimulants, the basal phosphorylation levels of important signalling molecules did not deviate much in haemocytes from acute myeloid patients when compared with the healthy ones. However, only when the patient blood samples were tinkered with a number of growth factors and cytokines, the signalling profiles corroborate with the tumour genetics, response to therapy and disease outcome (Finch et al. 2012). Induction with stimuli is important because many important signalling responses, like activation of ERK2 in response to epidermal growth factor receptor (EGFR), are initiated only when there is a drastic fold change and do not depend on the varying basal protein levels (Buscà et al. 2016).

11.5.3 Role of Perturbations in Cancer Networks

Network perturbations in a system due to both intrinsic states and extrinsic signals demonstrate its dynamic nature. Factors like cell lineage (affected by epigenetic changes and differential expression of genes), genetic background (varying individual to individual) and exogenous signals (like food, nutrients) can determine network adaptations. Cumulative integration of these internal and external influences eventually determines the emergence of healthy or diseased individuals. In cancer, it is necessary to understand the network perturbations or adaptations as they may have profound impact on patients' response to various drug-induced treatments and targeted therapies. Like in recent targeted therapy approach of BRAF inhibitors, application of Gleevec and Herceptin for chronic myelogenous leukaemia and breast cancer, respectively, exhibited variable responses to the drugs even for the patients sharing similar tumour types (Torti and Trusolino 2011).

For the understanding and quantification of such network activity and response, experiments must be designed so as to mimic the biological perturbations caused by any external stimuli or drugs. These kinds of responses also involve a large number of feedback loops. So, the key step in designing those experiments is to incorporate the data derived from the sample with the appropriate stimuli and measure their effects on feedback mechanisms. For example, transcriptional response to EGF stimulation results in the negative feedback factors like dual-specific phosphatases 10 and 16 (DUSP10 and DUSP16), which in turn terminate the same pathways that activate EGF target genes (Finch et al. 2012). Similarly, there can be various attenuating as well as escalating factors that can further determine the feedback scenario in a network, especially related to cancer. Thus, these feedback factors can be applied to design a model describing the algorithm or the sequence of events starting from the initial detection of an input to the response of the tumours towards any stimulus or drug. These network models can then be further used as templates to develop novel drugs as well as new combinations of already existing drugs.

11.5.4 Tools for *De Novo* Cancer Network Construction

Network frameworks generated from aforementioned approaches can form the basis for de novo construction of cancer networks. Some of the popular network construction pipelines are GeneMANIA (Montojo et al. 2014), MEMo (Ciriello et al. 2013), ReactomeFIViz (Fabregat et al. 2018), STRING (Jensen et al. 2009) and HyperModules (Leung et al. 2014). GeneMANIA and ReactomeFIViz are Cytoscape that use a series of algorithms such as identification of relevant genes or proteins, incorporating them in reference pathways, clustering and annotating the interacting partners and relating these clusters to tumour phenotype. These tools have been instrumental in identifying various prognostic biomarkers for ovarian, gallbladder and breast cancers (Jiang and Xu 2009; Fu and Wang 2013). Mutually Exclusive Modules (MEMo), as the name itself suggests, identifies mutual exclusivity of oncogenic alterations in groups of genes/proteins across different cancer data samples and groups them into subnetworks on the basis of functional properties such as severity, synthetic fatality, etc. Another module-based algorithm named HyperModules also locates subnetworks with cancer mutations that are responsible for tumour development or recurrence. Unlike MEMo, it also determines tumour subtypes and performs prediction analysis on patient survival rate based on different stages of cancer. HyperModules along with MEMo has been applied to study the kinase-signalling network in ovarian cancer and revealed network modules with mutations in phosphorylation sites and kinase domains that significantly correlated with patient survival.

11.5.5 Visualisation Tools for Cancer Networks and Pathways

Constructed networks when incorporated with the directionality of reactions and molecular interactions form pathways. Cancer pathways are complex, and their visualisation can be represented in a simple or static way like in the form of images or PDF files. Most of the pathway resources or databases like KEGG (Kyoto Encyclopedia of Genes and Genomes), PANTHER (Protein ANalysis THrough Evolutionary Relationships), WikiPathways, etc. provide data in these formats (Kanehisa et al. 2016; Thomas et al. 2003; Slenter et al. 2018). However, there is a second mode of visualisation that enables user to view pathways in a more interactive manner where they can integrate their own data in those pathways and put them into desired formats. For example, the web interface of KEGG-based pathway visualisation tool for cancer networks and pathways provides colour-coded shades for different gene and drug targets (related to cancer) according to their expression level in different disease conditions. The colour-coded shades range from blue to red with increase in the numerical values of their expression level. Some of the other KEGG-based interfaces such as BioCyc, MetaCyc and MEGU allow cancer biologists to map multiple layers of genomic and proteomic data simultaneously as well as overlay experimental data on the pathway maps by importing datasets from databases (Caspi et al. 2016; Kono et al. 2006). Other pathway resources like Reactome

(contains a front-end visualisation tool named after it) and WikiPathways use their own standalone analysis tool called PathVisio for interactive visualisation (Fabregat et al. 2018). These tools, with integrated packages like PathView written in R programming language and javascripts (BioJS), can also perform calculation of various ligand- and drug-induced effects on reference pathways along with customised visualisation (Luo and Brouwer 2013; Gómez et al. 2013).

11.5.6 Standard File Formats for Cancer Network Input and Output

While working with network visualisation tools, one must have the knowledge of various formats of the input data. Most of the modern network graph viewers come with their own input file formats to load and store the networks. Varieties of file formats and standard languages have been introduced to store and interpret biological information, which in turn are being used in cancer network and pathway visualisation. One of the most common and user-friendly languages for data visualisation is XML (Extensible Markup Language). Some of its features include platform independence and storage of information in the form of hierarchical tree structures that are readable by both humans and machines (Pavlopoulos et al. 2011). However, one disadvantage of XML is that due to tree-based topology, redundancy is imminent and might affect application efficiency due to higher storage, transmission and processing costs. To overcome redundancy, many open-source XML-based languages have been developed, which are more efficient in handling pathway complexities as they rely on layer-based approaches. Using layer-based approaches, users can specify the layer or level that represents their set of biological data, thus avoiding unnecessary calculations involving non-related layers. Some of the common languages that follow this method include BioPAX (Biological Pathway Exchange) (Demir et al. 2010), PSI-MI (Proteomics Standards Initiative Interactions) (Isserlin et al. 2011), CellML (Cell Markup Language) (Lloyd et al. 2008) and SBML (System Biology Markup Language) (Hucka et al. 2003). Among them, BioPAX spans the broadest range of biological data involving interactomes, small molecules and genetic interactions. PSI-MI is more specialised in proteomic data that are related to molecular interactions as well as regulatory and metabolic pathways. SBML, as the name suggests, is ideal for detailed study of the interacting partners, mostly during simulations. All these languages are also well suited for handling different kinds of cancer data and their visualisation in signalling pathways.

11.6 Conclusion

In-depth analysis of signalling pathways and networks can effectively reveal biological systems that are dysregulated in tumour cells. However, information available for pathways and networks both in normal and cancer cells is far from complete. Approaches like molecular modelling, high-throughput docking, simulations,

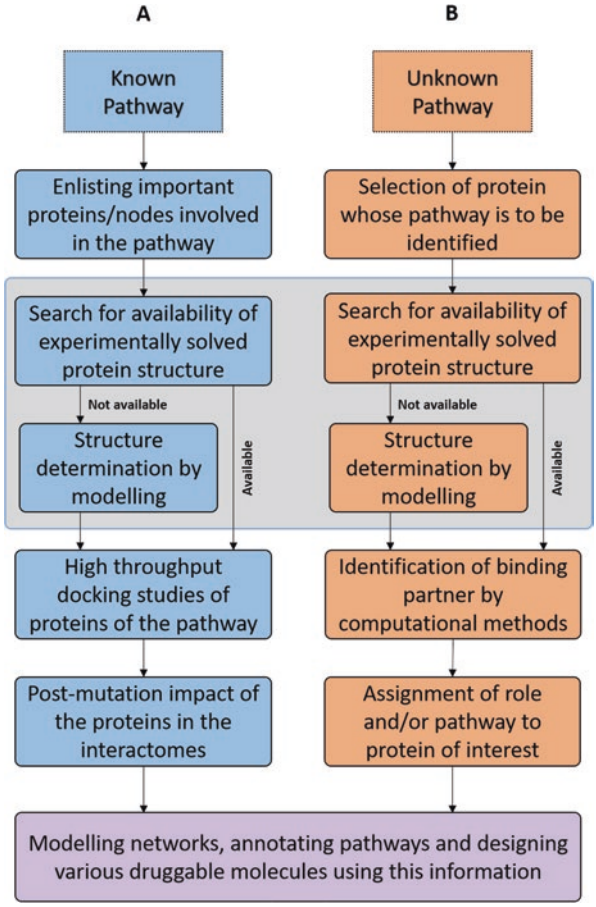

Fig. 11.3 Structural bioinformatics approach for cancer pathway analysis. General strategy involving molecular modelling, high-throughput docking, mutation detection and network modelling for analysing both known (*A*) and unknown (*B*) cancer pathways

network construction, interactome analysis, etc. require high-resolution structural data, accurate and detailed pathway descriptions with regulatory relationships and deep quantitative omics data. Moreover, biological network estimation using statistical and permutation-based methods is computationally expensive and time-consuming. With the increase in the number of reference molecules, pathways and networks, complexities are increasing day by day, demanding more powerful and efficient computational approaches (Fig. 11.3).

Another challenge faced by researchers while studying cancer network is to understand the role of mutations. Phenotypically, a mutation can either enhance or suppress a tumour depending on the cellular state and presence of other interdependent mutations alongside. So, it is important to establish annotation tools that can incorporate these conditions during analysis of different signalling pathways. The most important purpose of cancer pathway analysis is to develop pathway-specific therapeutics for treating rapidly evolving tumour cells. Development of quantitative models of tumour networks should reveal the role of these therapies on cancer

patients and will enable us to find out the right amount of dosage and drug combination that must be administered for the best possible outcome. With the advent of more advanced structural bioinformatics techniques, we believe that our understanding of cancer biology through the lens of pathway and network analysis will improve and transform our thinking on disease aetiology and treatment.

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Targeting Cancer from a Structural Biology Perspective

12

Derion Reid and Carla Mattos

Abstract

Cancers result from complex evolutionary processes with selective pressure leading to the accumulation of multiple mutations in proteins involved in the signaling circuitry and basic functions of the cell. Together, the mutant gene products co-opt the cell signaling programs that maintain homeostasis in normal tissues to serve the uncontrolled proliferation, survival, angiogenesis, metabolic, and migration functions that allow cancer cells to thrive at the expense of the organism. The details of how this happens are still not entirely understood. However, intense research in the last two decades has revealed a common set of hallmark traits acquired by cells in the progression to all cancers. A seminal article by Hanahan and Weinberg in 2000 (Hanahan and Weinberg 2000) first identified six hallmarks, with an update a decade later in 2011 (Hanahan and Weinberg 2011) that provided more detailed understanding of the original hallmarks and added two emerging new ones. It also identified enabling characteristics that facilitate acquisition of the hallmarks by cancer cells. In the present chapter, we briefly summarize the hallmarks of cancer and use the Hanahan and Weinberg framework to exemplify targets in signaling pathways that lead to each of the hallmarks from a structural biology perspective. We summarize the current state of inhibitors for at least one major target protein in each of the hallmark signaling circuitry.

Keywords

Hallmarks of cancer · Protein structures in cancers · Structure based inhibition

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12.1 Introduction

Cancers result from complex evolutionary processes with selective pressure leading to the accumulation of multiple mutations in proteins involved in the signaling circuitry and basic functions of the cell. Together, the mutant gene products co-opt the cell signaling programs that maintain homeostasis in normal tissues to serve the uncontrolled proliferation, survival, angiogenesis, metabolic, and migration functions that allow cancer cells to thrive at the expense of the organism. The details of how this happens are still not entirely understood. However, intense research in the last two decades has revealed a common set of hallmark traits acquired by cells in the progression to all cancers. A seminal article by Hanahan and Weinberg in 2000 (Hanahan and Weinberg 2000) first identified six hallmarks, with an update a decade later in 2011 (Hanahan and Weinberg 2011) that provided more detailed understanding of the original hallmarks and added two emerging new ones. It also identified enabling characteristics that facilitate acquisition of the hallmarks by cancer cells. In the present chapter, we briefly summarize the hallmarks of cancer and use the Hanahan and Weinberg framework to exemplify targets in signaling pathways that lead to each of the hallmarks from a structural biology perspective. We summarize the current state of inhibitors for at least one major target protein in each of the hallmark signaling circuitry.

Mutations occur frequently in normal cells, and there are myriad mechanisms to prevent deleterious changes to have a long-term effect. This includes a variety of DNA damage sensor and repair proteins (Polo and Jackson 2011) as well as signals that promote an apoptotic state resulting in cell death if the damage is severe or irreparable (Houtgraaf et al. 2006). Under normal circumstances, cell proliferation is highly regulated and occurs prominently during the wound healing process or in developmental stages of the organism where stem cells differentiate into the various tissue types (Vermeulen et al. 2003). It is kept in check by a series of antiproliferative signals propagated by tumor suppressor proteins that regulate the cell cycle (Sun and Yang 2010). Cancer cells acquire stemlike properties and promote gene expression of developmental proteins normally silenced in the adult, bypassing communication with neighboring cells via the extracellular matrix essential in the maintenance of homeostasis (Micalizzi et al. 2010). In general, the hallmarks of cancer comprise a collection of functions that are acquired and combined to usurp the cell's regulatory mechanisms and promote a highly proliferative state. These functions are not necessarily acquired in a particular order but are a result of random mutations that occur at high frequency due to genomic instability driven by uncontrolled proliferation, with selection of mutants that lead to the survival of the fittest. In this sense, cancer is a collection of diseases where the process that is at play to promote the fittest organisms over its competitors in a particular environment at the species level takes over at the cellular level in such a way that the cancer cells survive to the detriment of the surrounding normal cells and eventually the individual organism.

In brief, there are six well-established hallmarks of cancer as summarized by Hanahan and Weinberg (2011): sustaining proliferative signaling, evading growth

suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. Emerging hallmarks include deregulating cellular energetics and avoiding immune destruction. Genomic instability and chronic inflammation are enablers that accelerate the evolutionary process toward cancer and maintenance of cancer traits. Each of the hallmarks is a result of mutations in master signaling proteins that normally function under tight control, with the result that they are activated, deactivated, or functionally changed to select for one or more of the hallmarks. In many cases, the structural biology of the proteins involved in cell signaling circuits usurped in cancers has provided valuable insight into the molecular mechanisms through which they function and are coopted (Nussinov et al. 2013). Many, such as the growth factor receptors and a large number of kinases are targets for drugs against cancers. Others, such as Ras mutants, have been so far "undruggable," but their inhibition is predictive of a huge step forward in the treatment of cancers and sustained efforts to drug Ras proteins are ongoing, particularly for K-Ras (Marcus and Mattos 2015). Although many of the current drugs in the clinic are successful in temporarily halting the progression of late-stage tumors, almost always the cell develops resistance to the drug and the tumors return undaunted. This is due to the high rate of mutation and adaptation conferred by the hallmark traits in an environment of genomic instability and inflammation. It is increasingly apparent that the best chance forward is to selectively combine drugs to simultaneously target proteins in signaling pathways associated with several of the hallmarks in order to minimize alternative pathways for adaptation and resistance. Hanahan and Weinberg make this point eloquently, and Fig. 12.1 echoes their representation of possible inhibition strategies. Below we summarize the traits of each hallmark and focus on the structural biology and drug molecules for selected target proteins in each of the inhibitor classes that they describe (Hanahan and Weinberg 2011).

12.2 Sustaining Proliferative Signaling

The first of the hallmarks of cancer is the acquisition of mutations that allow cells to sustain proliferative signaling, becoming insensitive to the normal tight control that regulates the cell cycle through the timely release of growth-promoting signals. The release of growth factors is thought to result from communication between cells through the extracellular matrix. Cells with mutations in proteins that become insensitive to growth factors proliferate constitutively and may go on a path to neoplasia if unchecked. Growth factors in general bind to receptor tyrosine kinases on the cell surface, promoting dimerization/oligomerization of the receptor and activation of the intracellular kinase domain (Lemmon and Schlessinger 2010). This results in propagation of several signal transduction cascades that together promote cell proliferation and survival. Although several receptors can participate in the maintenance of a proliferative state, we focus here on the particular example of the epidermal growth factor receptor (EGFR) known to

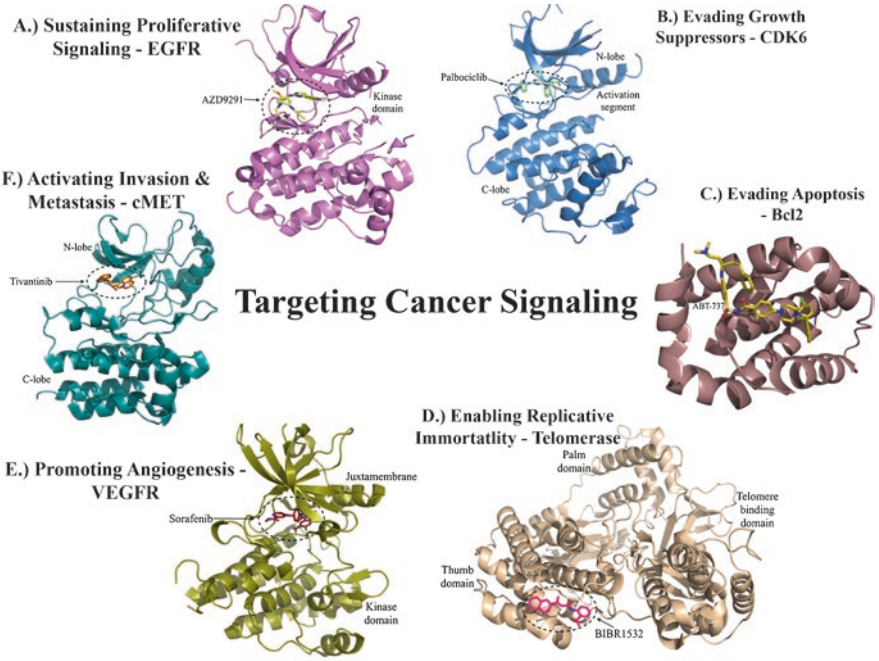

Fig. 12.1 The hallmarks of cancer with associated target proteins. (**a**) The intracellular kinase domain of the EGFR receptor is targeted to prevent autophosphorylation and activation of proliferative pathways. (**b**) The cyclin-dependent kinase 6, targeted at the checkpoint of entering the cell cycle. (**c**) Bcl-2 bound to a BH3-only mimic to release BAX and BAK onto the membrane thereby promoting apoptosis. (**d**) The TERT domain of telomerase targeted to limit the cell's replicative potential. (**e**) The intracellular kinase domain of the VEGF receptor targeted to inhibit angiogenesis. (**f**) cMET kinase is inhibited in an attempt to inhibit the EMT program

activate the mitogenic pathway Ras/Raf/MEK/ERK leading to the expression of proteins that promote cell division and proliferation (Yewale et al. 2013). Independence from growth factor control can be achieved by overexpression of the receptor or by constitutively activating mutations in the receptor or in a signaling protein in the pathway, such as Ras or Raf. Indeed about 20% of all human cancers have activating mutations in Ras (Prior et al. 2012), and 50% of melanomas harbor mutations in Raf (Fattore et al. 2013).

12.2.1 The Epidermal Growth Factor Receptor (EGFR)

Receptor tyrosine kinases fall into four large families in humans: EGFR, ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4. In this section, we focus on EGFR, often found to be hyperactivated in human cancers and a major target in cancer

treatment, particularly for lung, breast, and glioblastoma cancers (Arteaga 2011; Mok 2011). Although traditional models show EGFR family functions via ligand-induced receptor dimerization, structural studies suggest it is also allosterically regulated (Endres et al. 2011; Ferguson 2008). The structure of human EGFR is composed of the N-terminal extracellular region containing four domains I–IV (residues 1–620), a single transmembrane (TM) region (residues 621–643), and the intracellular domains, including a juxtamembrane region (644–685) that together with the TM region stabilize the asymmetric activation of the kinase (Endres et al. 2011), the kinase domain with conventional N-lobe and C-lobe (Sicheri and Kuriyan 1997) (residues 686–953), and a C-terminal region (residues 954–1186) (Fig. 12.1a). The C-terminal region is implicated in autoinhibition of the kinase, with five tyrosines that can be phosphorylated and dephosphorylated as part of the kinase regulatory mechanism. EGFR is activated by seven different endogenous ligands, including EGF, TGF-α, ARG, and EGN, that bind to the extracellular domains of the receptor to promote dimerization and activation of the intracellular kinase domain (Schneider and Wolf 2009). Each ligand contains an EGF-like domain that can be cleaved from its membrane-bound peptide and bind EGFR to activate cell proliferation in an endocrine, paracrine, or autocrine fashion. The EGF ligand is a 53-amino acid polypeptide that binds between domains I and III of the extracellular region of EGFR, which in the unbound inactive state are separated from each other in space (Ogiso et al. 2002). EGF contains three loops (A, B, C) that are held together by disulfide bonds C6–C20, C14–C31, and C33–C42. Loop B makes specific interactions within a hydrophobic pocket on domain I of EGFR, while loops A and C interact at their respective binding sites on domain III (Ogiso et al. 2002). These interactions promote a conformational change from a tethered structure, where domain II dimerization elements are tucked into domain IV, to an extended form stabilized by EGF bringing domains I and III close together to expose the dimer interface in domain II (Ferguson 2008; Ogiso et al. 2002). Dimerization activates the EGFR receptor, with one kinase domain remaining inactive while stabilizing the active kinase conformation of the other (Endres et al. 2011).

Inhibitors of EGFR currently in the clinic use two major strategies (Harari 2004; Yewale et al. 2013): the monoclonal antibodies (e.g., cituximab) bind to the extracellular domain III, impeding binding of EGF and other activating ligands and preventing dimerization of the receptor (Garrett et al. 2002, Ogiso et al. 2002), and the tyrosine kinase inhibitors compete with ATP (Fig. 12.2) to prevent in trans autophosphorylation that leads to activation of several signaling pathways associated with proliferation and survival (Stamos et al. 2002). Immunotoxin conjugates that deliver toxins (Azemar et al. 2000), antisense nucleotides (Ciardiello et al. 2001) or iRNA (Yamazaki et al. 1998) that decrease the expression of EGFR, have also been tried in the past as strategies to inhibit EGFR activity. The tyrosine kinase inhibitors are the best characterized with high-resolution crystal structures, opening the possibility for structure-based inhibitor optimization. A result of this approach is the

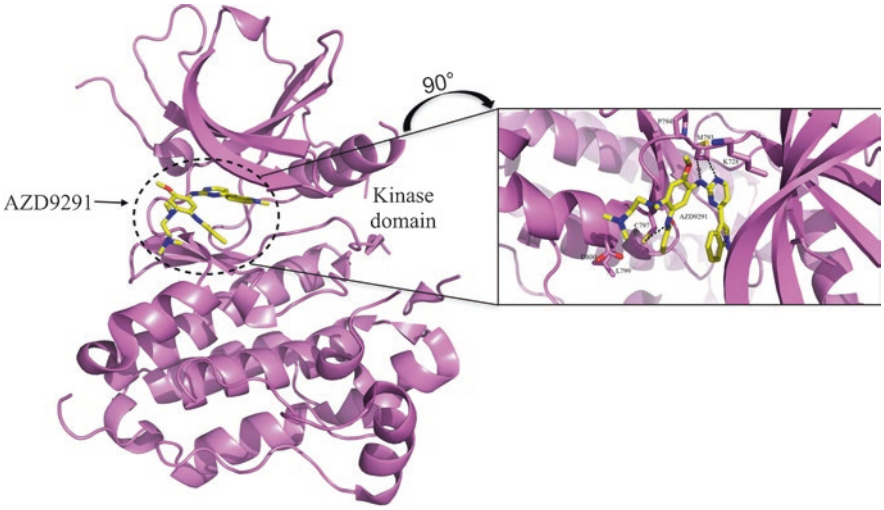

Fig. 12.2 AZD9291 bound to EGFR. AZD9291 (yellow) binds in the ATP-binding pocket of EGFR (purple) between P794 and C797, with extensive van der Waals interactions and a few polar contacts

inhibitor mereletinib (AZD9291), shown in Figs. 12.1a and 12.2 in complex with the EGFR kinase domain.

12.2.2 Ras GTPases

Ras is a well-studied example of an oncogene that is involved in proliferative signaling of cancer cells. The discovery of Ras GTPases in 1982 due to its ability to activate and transform human cancer cell lines has led to much effort to understand its functionality via structural, biochemical, and cellular studies (Reddy et al. 1982). Ras proteins are in a crucial position in signaling cascades that become proficient in sustaining proliferation in cancerous cells. They are activated by multiple upstream signals, including those transduced by EGFR, and control multiple signaling pathways (Nussinov et al. 2013). Ras GTPases function via a binary switch mechanism, where signaling is propagated when Ras is bound to GTP and stopped when bound to GDP. The nucleotide-bound state is tightly regulated by GEFs and GAPs (Bos et al. 2007). Some Ras oncogenic mutants, such as those at the G12 (P-loop) and Q61 (switch II) residues, evade regulation by impairing hydrolysis of GTP, while others such as G13D (P-loop) and A146T (near the nucleotide guanine base) constitutively exchange GDP for GTP (Haigis 2017). In either case, the end result is sustained high levels of Ras-GTP independent of growth factors, promoting uncontrolled cell proliferation. K-Ras is the most frequently

mutated in cancers among the three major isoforms of Ras (K-Ras, N-Ras, H-Ras) (Prior et al. 2012).

Each isoform of Ras GTPase contains a catalytic G-domain (residues 1–166) and a C-terminal hypervariable region. The G-domain of Ras is a Rossmann fold consisting of a six-stranded β-sheet and five α-helices, where α-helices 2, 3, and 4 are on one side of the β-sheet and α-helices 1 and 5 lay on the other side (Raimondi et al. 2010). The G-domain of Ras forms a parallel/antiparallel β-sheet mix unlike a standard Rossmann fold motif (Pai et al. 1990). The modified triple-layered αβα sandwich is sequentially found as β1-α1-β2-β3-α2-β4-α3-β5-α4-β6-α5 with intervening loops between the secondary structural elements (Johnson and Mattos 2013).

The catalytic G-domain is divided into two lobes: the effector lobe made up of β1-α1-β2-β3-α2-β4 and the allosteric lobe consisting of α3-β5-α4-β6-α5. The effector lobe of Ras is 100% conserved among the three isoforms, making up the active site containing the phosphate-binding loop (residues 10–17), switch I (residues 30–40), and switch II (residues 60–76) (Gorfe et al. 2008; Johnson et al. 2017). As indicated by its name, the effector lobe of Ras is responsible for propagating its signal via interacting with effector and regulator molecules based on the nucleotide bound in the active site (Vetter and Wittinghofer 2001). The crystal structures of Ras in complex with many of its partners have been solved, such that we have some understanding of binding mechanism involving these complexes. Ras in complex with the GEF Sos shows a very open nucleotide-binding pocket from which Mg^{2+} is expelled, making it clear that the exchange factor operates by opening the active site and weakening the interaction of Ras with GDP and its coordinating Mg^{2+} ion (Boriack-Sjodin et al. 1998), such that the nucleotide can diffuse out. GTP then binds to the active site, promoting Ras interaction with various effector molecules including Raf, PI3K, and NORE1A. The structures of these effectors in complex with Ras have been determined, showing interactions through the effector lobe interface, which is disordered in uncomplexed Ras and ordered specifically to complement each effector protein (Fetics et al. 2015; Pacold et al. 2000; Stieglitz et al. 2008). The complex with GAP also shows interaction in the effector lobe, with an interface that spans switch I and switch II, and the GAP arginine finger placed over the nucleotide to stabilize the transition of the GTP hydrolysis reaction (Scheffzek et al. 1997).

The allosteric lobe (residues 87–171) is 90% conserved between the Ras subfamilies and accounts for isoform-specific differences between K-Ras, N-Ras, and H-Ras in terms of G-domain biochemistry and conformational states (Johnson et al. 2017; Parker et al. 2018). The allosteric lobe of Ras interacts with the membrane (Gorfe et al. 2007) and is followed by the C-terminal hypervariable region, which is essential in localizing Ras to various sites of the membrane microdomains (Parker and Mattos 2015). More recently, the allosteric site found at helix 3/loop 7 of the allosteric lobe has been hypothesized to play a role in regulating intrinsic hydrolysis in the presence of Raf (Buhrman et al. 2010).

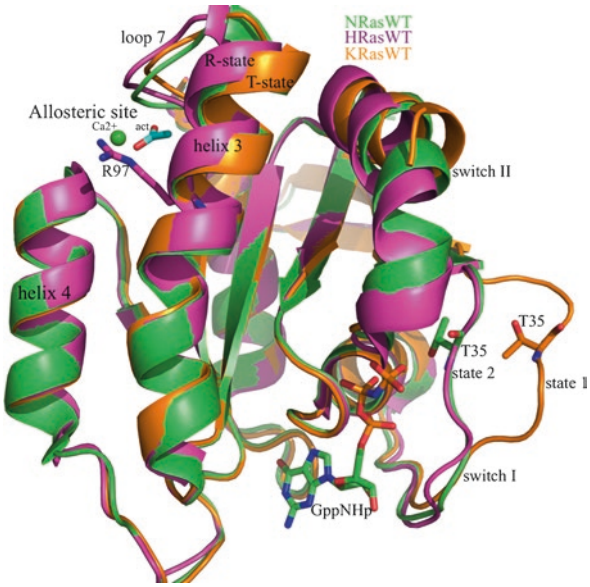

Fig. 12.3 Conformational states in GTP-bound Ras. Switch I interconverts between an open (state 1) conformation that favors nucleotide exchange and a closed (state 2) conformation associated with effector binding and GTP hydrolysis. The Ras isoforms have different preferences for these states. In the presence of Raf (state 2), crystals of H-Ras show bound calcium and acetate in the allosteric site. This results in a shift of helix 3/loop 7 away from switch II, with a change in the conformational equilibrium from the T-state (disordered active site) to the R-state (ordered active site) and placement of Q61 for hydrolysis of GTP

Ras samples a variety of conformational states that affect its ability to hydrolyze GTP (Fig. 12.3). For example, state 1 favors nucleotide exchange where Y32 and T35 of switch I are swung away from the active site, thus rendering an open active site and making it difficult for effector molecules to bind (Spoerner et al. 2001). The first structure of H-Ras-GppNHp was determined in the early 1990s from crystals with symmetry of the space group $P3_221$ (Pai et al. 1990). The switch I in this structure superimposes well with the conformation of switch I in GAP-bound Ras-GppNHp (Scheffzek et al. 1997). Crystals with symmetry of space group R32 were discovered more recently (Buhrman et al. 2010). This crystal form results in a structure where switch I resembles that in the Ras/Raf-RBD complex (Fetics et al. 2015). In both structures, T35 interacts with the active site Mg^{2+} ion, and in the latter, the active site is "closed" with Y32 over GTP, in a state 2 conformation that appears to favor intrinsic hydrolysis on Ras. An open state 1 conformation, with both Y32 and T35 away from the nucleotide, is seen in the T35A and T35S mutants of H-Ras, as well as in the wild-type K-Ras bound to GTP analogs (Fig. 12.3) (Araki et al. 2011; Parker et al. 2018). The Mattos Lab has hypothesized an allosteric switch mechanism of Ras that plays a role regulating intrinsic hydrolysis in the presence of Raf (Buhrman et al. 2010). In this model, a ligand, mimicked by calcium and acetate in

crystal structures, binds near the Ras-membrane interface shifting helix 3, loop 7, and the C-terminal end of switch II toward helix 4 to promote a hydrogen bonding network. Raf is a unique effector protein that only binds switch I of Ras, whereas all other effectors bind both switch I and II. This orders switch II and places the Q61 catalytic residue into the active site for catalysis (Buhrman et al. 2010). Interestingly, dimers of Ras appear in both the Ras structure (PDB ID 3K8Y) and in the Ras/Raf-RBD complex (PDB ID 4G0N), such that we suspect that binding of Ca^{2+} occurs in the context of the dimer, linking dimerization and intrinsic hydrolysis activated by an allosteric switch mechanism. We have shown that the binding of Raf-RBD to Ras promotes ordering of the Ca^{2+} binding allosteric site, which we postulate triggers intrinsic hydrolysis to turn off the signal through Ras/Raf (Fetics et al. 2015). In comparison, the RasQ61L/Raf-RBD structure stabilizes switch II in an anticatalytic conformation while increasing dynamics in the allosteric site (Fetics et al. 2015). This combination of effects is expected to shut down intrinsic hydrolysis in the Q61L mutant, consistent with our kinetic analysis of the complex for this mutant (Buhrman et al. 2007).

For many years, it was assumed that Ras proteins shared the same function with different localization properties, because these proteins have only small variations in their sequence, mainly in sites of interaction with the cell membrane to which the catalytic G-domains are tethered via their highly divergent lipidated HVRs. Of the three major Ras proteins (H-Ras, K-Ras, and N-Ras), N-Ras is the least studied. Biochemical work in the late 1980s suggested that wild-type H-Ras and N-Ras have distinct intrinsic hydrolysis rates, hinting that there may be differences between the Ras isoforms (John et al. 1988; Neal et al. 1988). It was recently confirmed that the G-domains of the three Ras proteins are indeed biochemically distinct (Johnson et al. 2017). Consistently, the rates of particular Ras mutants associated with specific cancers vary significantly (Prior et al. 2012), and reasons for this are currently the topic of intense investigation. K-Ras is the most commonly mutated isoform and is most susceptible to mutations in pancreatic tumors, whereas N-Ras is more susceptible to be mutated in hematopoietic tumors or melanomas. The recent crystal structures of wild-type N-Ras and K-Ras bound to GTP analogs have revealed important differences in conformational states between the isoforms that can partially explain their biochemical differences (Fig. 12.3) (Johnson et al. 2017; Parker et al. 2018). They also add valuable information that can be used in the development of specific inhibitors based on stabilization of certain conformations in the oncogenic variants (Kauke et al. 2017). The structural work on Ras proteins clearly shows that different mutants have unique effects on the structure and dynamics of each isoform (Johnson et al. 2019). These effects may be linked to the selection of particular mutants in specific cancer types (Prior et al. 2012).

Although there are no drugs targeting Ras in the clinic, there is intense effort to find ways to inhibit Ras signaling, particularly K-Ras (Marcus and Mattos 2015), and the wealth of structural information currently available is likely to expedite the process of drug discovery. The mapping of the binding surface of Ras was done using the multiple solvent crystal structures (MSCS) method for identifying sites of

protein-protein or protein-ligand interactions and shows several possible binding pockets for targeting Ras (Buhrman et al. 2011). The most prominent site, between switch II and α3, binds small molecule covalent inhibitors of K-RasG12C, commonly mutated in lung adenocarcinoma (Gentile et al. 2017; Ostrem et al. 2013). Other Ras inhibitors attempt to disrupt nucleotide exchange by GEFs to maintain Ras in an inactive state (Sun et al. 2012). Most recently, it has become clear that Ras functions through dimerization, at least in the proliferative pathway through Raf (Ambrogio et al. 2018), and efforts are ongoing to develop inhibitors such as NS1, which targets the allosteric lobe and is selective for H- and K-Ras (Spencer-Smith et al. 2017). The structure of the complex between H-Ras and NS1 shows that the inhibitor binds at the α4–α5 dimer interface with cell biology work supporting robust inhibition of Ras/Raf/MEK/ERK. This validates the dimer interface as an exciting area to target Ras in the treatment of cancers.

12.3 Evading Growth Suppressors

The cell cycle is clocked by a tightly regulated series of events coordinated by temporally varying concentrations of specific cyclins that activate cyclin-dependent kinases (Ckds) to ensure systematic progression of the cell toward mitosis and cell division in response to growth factors (Morgan 1997; Malumbres 2014). The tumor suppressor retinoblastoma protein (RB protein) is a major regulator of cell cycle progression as, in synergy with TP53, it integrates various extracellular and intracellular signals to decide whether or not a cell should go through its growth and division cycle (Burkhart and Sage 2008). Thus, the tumor suppressor proteins provide a major defense mechanism against the uncontrolled cell proliferation associated with cancers (Hanahan and Weinberg 2011). Mutations in RB and TP53 provide a common mechanism through which cancer cells evade the control mechanisms that suppress the cell cycle. Defects in tumor suppressor pathways promote progression to mitosis, and thus, a major strategy to overcome these defects is the development of cyclin-dependent kinase inhibitors (Kalra et al. 2017).

Cyclin-dependent kinases are serine/threonine protein kinases that are central players in the regulation of the cell cycle through their interactions with cyclins to regulate transcription of genes involved in DNA synthesis, centrosome maturation and separation, chromosome condensation, as well as entry into the mitotic phase after breakdown of the nuclear envelope (Malumbres 2014). Mammalian cells have eight subfamilies of Cdks, three of which comprise proteins involved in the cell cycle that interact with cyclins, whose concentrations change during the various phases of the cell cycle, and the remaining five subfamilies of Cdks are involved in transcription and their associated cyclins are not subject to the oscillations timed with the cell cycle. Cdks and cyclins are regulated by a combination of gene expression and protein degradation, and in many cases through protein localization, ensuring that the four phases of the cell cycle (G_1, S, G_2, and M) are tightly regulated and go through checkpoint mechanisms where Cdks/cyclins are inhibited by cyclin-dependent kinase inhibitors (CDKIs) to ensure faithful DNA replication (G_1/S) and division

(G_2/M) (Malumbres 2014; Kalra et al. 2017). When DNA damage is detected, anti-growth factors are activated to inhibit cellular growth by either allowing the cell to enter a state of senescence or promoting an apoptotic state (Weinberg 1995). When unphosphorylated, RB proteins are active and inhibit the E2F transcription factors required for the transcription of genes needed for DNA synthesis (Dyson 2016). Cdk4 and Cdk6, activated by cyclin D, phosphorylate the RB protein, thereby releasing E2F and entry into the cell cycle. In order for cancer cells to constitutively proliferate, they must evade tumor suppressors. Under these circumstances, inhibition of Cdks/cyclins provides an opportunity in the war against cancer, and this topic is a subject of recent reviews (Kalra et al. 2017; Li et al. 2016; Roskoski 2015).

The core structure of Cdks is typical of kinases, composed of a small β-sheet N-terminal lobe that also contains the C-helix and a larger α-helical C-terminal lobe containing the activation loop with a phosphorylation site (Fig. 12.1b) (Welburn and Jeyaprakash 2018). The ATP-binding pocket resides at the interface between the two lobes, and the activity of the kinase depends on conformational changes involving the C-helix and the activation loop. The monomeric structure of Cdk2 shows the C-helix turned away from the active site and the activation loop blocking ATP-binding pocket in the inactive conformation of the kinase (Schulze-Gahmen et al. 1996). Cyclin A is composed of two 5-helix bundles, one of which interacts intimately with the N-terminal lobe of Cdk2 (Echalier et al. 2010). The binding of cyclin A positions the C-helix such that catalytic residues are in place in the active site and stabilizes the activation loop in an open conformation, where it is simultaneously removed from the active site and exposed to solvent such that T160 can be phosphorylated, stabilizing the kinase in its active form (Russo et al. 1996). Variations on the remaining interactions among various Cdks and their cyclin partners give a wealth of functional specificity that can be explored to guide drug development (Echalier et al. 2010; Welburn and Jeyaprakash 2018; Kalra et al. 2017).

One example of a Cdk/cyclin inhibitor approved by the FDA and currently in the clinic is palbociclib, which binds CDK4/6 in complex with cyclin D, thereby inhibiting entry into the cell cycle. This inhibitor binds CDK4/cyclin D and Cdk6/cyclin D with an IC_{50} of about 10 nM and with very high selectivity, as demonstrated by IC_{50}'s greater than 10 μM for 36 other tested kinases (Lu and Schulze-Gahmen 2006). Chemically, it is a pyrido[2,3-*d*]pyrimidin-7-one scaffold that binds tightly into the ATP-binding pocket of CDK4/6 and is coordinated by D163 and V101. Binding of palbociclib disrupts phosphorylation of retinoblastoma proteins inhibiting cell division in G1 (Fry et al. 2004). The crystal structure of palbociclib in complex with Cdk6/Cyclin D shows the inhibitor bound in the ATP-binding pocket between the N- and C-terminal lobes of the kinase, making extensive interactions, beyond that observed for less selective inhibitors (Fig. 12.4) (Lu and Schulze-Gahmen 2006). Although a structure of palbociclib in complex with Cdk4/cyclins D is not available, the crystal structure of the ATP-bound complex suggests a similar mode of interaction with Cdk4 (Day et al. 2009). Selectivity is a challenge faced in the development of kinase inhibitors in general and for inhibitors of Cdks/cyclins in particular. However, examples such as palbociclib provide an incentive to continue to search for potent and selective inhibitors against kinases involved in human cancers.

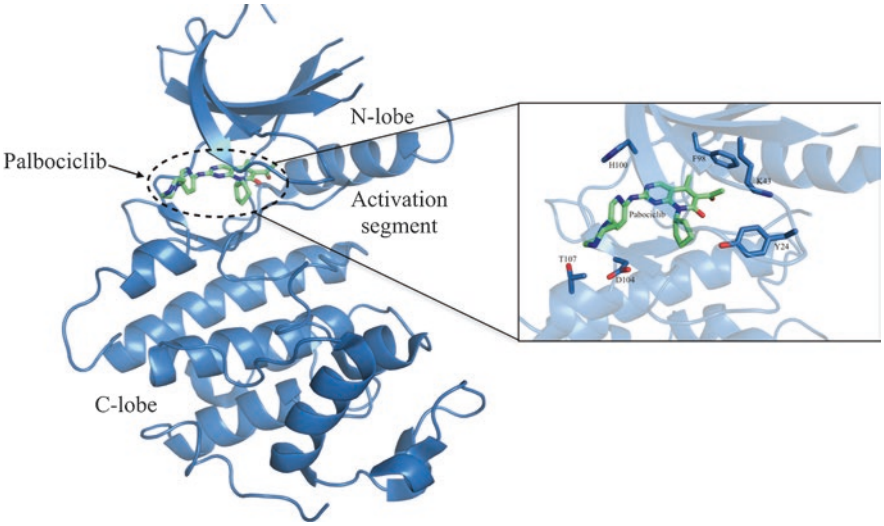

Fig. 12.4 Palbociclib bound to CDK6. Palbociclib is a pyrido[2,3-*d*]pyrimidin-7-one scaffold that binds tightly into the ATP-binding pocket of CDK4/6. The nature of the interactions within this pocket differs significantly from those shown in Fig. 12.2 for EGFR

12.4 Evading Apoptosis

Another important hallmark is the acquired ability by cancer cells to evade apoptosis. In normal cells, the overexpression of oncogenes, such as Ras, Raf, and Myc, extensive or irreparable DNA damage, and other cell stress-promoting events lead to senescence and/or apoptosis (Collado and Serrano 2010; Evans et al. 2009). This is triggered by abnormality sensors that activate the major tumor suppressor proteins RB and TP53 (Adams and Cory 2007; Lowe et al. 2004), gatekeepers that integrate signals via the extrinsic and intrinsic pathways to determine whether the cell needs to enter the apoptotic state (Khan et al. 2014). Once the cell is committed to apoptosis, initiator and executioner cysteine-specific aspartate proteases (Caspases) are activated to destroy the cell from within, carrying the process to its end (MacKenzie and Clark 2012).

The extrinsic pathway of apoptosis is activated upon the binding of a death receptor (DR) ligand. Death receptors are part of the tumor necrosis factor receptor superfamily, composed of TNF-R1, Fas (apo1), and DR3 (Khan et al. 2014; Yan and Shi 2005). In response to extracellular signals due to stress or morphological changes, a pro-apoptotic ligand, such as FasL, TNF, or TNF-related apoptosis-inducting ligand receptors (TRAIL), binds to DR inducing trimerization (Yan and Shi 2005). Once the DR is activated, Fas-associated death domain protein (FADD) and caspases 8 and 10 interact at the intracytoplasmic tail of the DR. This forms a death-inducing signal complex (DISC) at the plasma membrane, which activates initiator caspases, ultimately leading to activation of the executioner caspases 3 and

7 and potent DNAse proteins involved in the apoptotic process (Khan et al. 2014). The extrinsic pathway has been targeted for cancer treatment to some extent, although most of the efforts to promote apoptosis in cancer cells have been focused on promotion of the intrinsic apoptotic pathway (Koff et al. 2015).

The intrinsic pathway of apoptosis is activated when an internal stimulus, such as DNA damage, is present. This pathway is tightly regulated via the Bcl-2 family of proteins (Koff et al. 2015). The Bcl-2 family is composed of pro-apoptotic proteins, such as Bax and Bak, and anti-apoptotic proteins such as Bcl-2, Bcl-X_L, Bcl-W, Bfl-1, and Mcl-1 (Reed 1994). The tumor suppressor TP53 controls the intrinsic apoptotic pathway involving the BCL-2 family of proteins (Delbridge and Strasser 2015), including Bax and Bak, which promote the release of cytochrome c by permeabilizing the mitochondrial membrane (Cosentino and Garcia-Saez 2017). The Bcl-2 family proteins contain anywhere from one to four Bcl-2 homology (BH) domains, BH1, BH2, BH3, and BH4, and can be divided into three groups: the anti-apoptotic proteins, the multi-BH domain pro-apoptotic proteins Bax and Bak, and the BH3-only pro-apoptotic proteins. The balance of interactions between these groups of proteins determines whether the cell will live or die. The anti-apoptotic proteins share the BH1-4 domains and are able to bind the BH3 domains of Bax and Bak, keeping them from activating membrane permeability.

The first crystal structure of a Bcl-2 family member was that of Bcl-X_L solved in the early 1990s (Muchmore et al. 1996). The protein has seven α-helices, where hydrophobic α5 and α6 are centrally located flanked by five amphipathic helices (Fig. 12.1c). Bcl-X_L contains three homologous domains BH1 (residues 129–147), BH2 (180–197), and BH3 (residues 90–98) in close proximity forming a hydrophobic binding pocket to interact with pro-apoptotic Bcl-2 family members such as Bax and Bak (Muchmore et al. 1996), with the Bcl-X_L BH1 and BH2 domains essential for heterodimerization with Bax (Yin et al. 1994). The overall fold of the multidomain Bcl-2 proteins highly resembles the fold of the membrane insertion domain of bacterial toxins where two helices are centrally located and are long enough to span the membrane (Muchmore et al. 1996; Parker and Pattus 1993). This similarity gave insight as to how apoptotic Bcl-2 proteins create pores in the cytoplasmic membrane to release cytochrome c or other mitochondrial proteins (Muchmore et al. 1996), a process that has gained substantial mechanistic insight based on more recent crystal structures (Cosentino and Garcia-Saez 2017). The pro-apoptotic proteins Bax and Bak are activated by a small set of α-helices known as BH3-only proteins that bind Bcl-2 anti-apoptotic proteins, thereby releasing the proteins involved in the first step leading to apoptosis. The crystal structure of Bak in complexed with Bcl-X_L shows that Bak binds in the BH1–3 cleft making extensive hydrophobic contacts at the interface. In the presence of an internal death signal, pro-apoptotic BH3-only proteins bind in the hydrophobic cleft of Bcl-2 anti-apoptotic proteins, releasing Bak and Bax, which are dephosphorylated, and a structural rearrangement leads to translocation to the mitochondrial membrane to facilitate apoptosis (Scorrano and Korsmeyer 2003; Ghobrial et al. 2005). The crystal structure of a BH3-only pro-apoptotic protein in complex with Bax has elucidated the mechanism of pore formation (Cosentino and Garcia-Saez 2017). The

binding of BH3-only proteins to Bax displaces the C-terminal α-helix, which inserts into the mitochondrial outer membrane, with several of these complexes coming together to form a 3D pore permeable to cytochrome c (Ghobrial et al. 2005). Cytochrome c in the cytoplasm binds to Apaf-1, a caspase adaptor protein, leading to subsequent activation of initiator caspase 9 forming the caspase recruitment domain or apoptosome that activates the executioner caspases 3 and 7 (Ghobrial et al. 2005).

Given the above mechanism, the hydrophobic cleft of Bcl-2 proteins has become a focused target for cancer therapies, and a significant number of mimetics of BH3-only proteins that bind anti- and pro-apoptotic proteins to promote apoptosis have been developed and tested in the clinic (Cragg et al. 2009; Delbridge and Strasser 2015). One example is ABT-737, a small molecule BH3-only mimic that inhibits anti-apoptotic Bcl-2 proteins, by binding in the hydrophobic BH3 groove of Bcl-2, Bcl-X_L, and Bcl-W, but has lower affinity for homologous proteins Mcl-1 and Bcl-B (Oltersdorf et al. 2005). The ABT-737 ligand is composed of a chloro-biphenyl or thio-phenyl moieties on the end that binds deep into the hydrophobic pocket of Bcl-X_L, and the crystal structure of its complex with Bcl-X_L shows the acylsulfonamide moiety of ABT-737 forming a hydrogen bond with Gly138 of Bcl-X_L (Fig. 12.1c) (Lee et al. 2007). A structure of Mcl-1 in complex with Bim, a BH3-only protein that regulates Mcl-1 activity, shows that the helix 3 of Mcl-1 where the C-terminus of the peptide ligand binds is more solvent exposed compared to Bcl-X_L, likely explaining the low affinity of the thio-phenyl moiety of ABT-737 for Mcl-1 (Czabotar et al. 2007). This makes ABT-737 a weak cytotoxic agent for cancers where Mcl-1 plays an important role in evading apoptosis, such as chronic lymphocytic leukemia, multiple myeloma, and B cell lymphoma (Czabotar et al. 2007). A large number of other BH3-only mimetics have been developed and are being tested. Although successful to some extent, the current issues with these drugs include a dose limit beyond which there is significant toxicity and the problem of fine-tuning specificity (Matsumoto et al. 2016). The efficacy of BH3-only mimetics at the low doses tolerated by patients appears to increase when given in combination with inhibitors of kinases in the proliferative pathway (Cragg et al. 2009).

12.5 Enabling Replicative Immortality

Normal cells can grow and divide to a limited extent before the ends of their chromosomal DNA, the telomeres, are depleted and the cell goes into either senescence, where they remain alive without the ability to grow, or crisis leading to apoptosis (Hanahan and Weinberg 2011). Telomeres are DNA-protein complexes localized at the end of chromosomes composed of repetitive TTAGGG sequences (Lingner et al. 1995). Every time DNA is replicated, a few nucleotide bases at the 3′ end of linear chromosomes are not replicated, such that with every replication cycle the DNA is a little shorter, with fewer telomeric repeat sequences (Chakhparonian and Wellinger 2003; Collins and Mitchell 2002). DNA that lacks sufficiently long telomere undergoes fusion with other pieces of genomic DNA, leading to genomic instability

followed by senescence or death (Smogorzewska and de Lange 2004; Blackburn 2001). The telomere serves as a protective cap at the end of the DNA, and its length determines the number of times the cell can divide.

Telomerase is a ribonucleoprotein capable of reverse-transcribing RNA, adding telomeric repeats to the ends of chromosomal DNA, thus extending the replicative potential of the cell (Kelleher et al. 2002). This enzyme is expressed during development and in proliferative tissues such as ovary and testicles, as well as the hematopoietic tissues, but is repressed in somatic tissues in the adult, such that most cells have a limited number of replicative cell cycles (Dong et al. 2005). Cancer cells usurp the telomerase system, promoting its expression to replenish the telomeres, rendering the cells immortal. This is an important hallmark of cancer that, together with the other traits, supports constitutive proliferation. Given the importance of telomerase activity for maintaining the cell's ability to divide, this enzyme has long been a key target for drug discovery in the treatment of cancer (Shay and Wright 2002; White et al. 2001).

The structure of telomerase consists of the protein catalytic unit telomerase reverse transcriptase (TERT) and a large telomerase RNA molecule (TR) that provides the template for extension of the 3′ end of DNA by reverse transcription (Autexier and Lue 2006). The TERT protein shows the classical conserved motifs that appear in reverse transcriptases within the context of the common domains associated with all nucleotide polymerases: the right hand with fingers, palm, and thumb (Lue et al. 2003; Wyatt et al. 2010; Mitchell et al. 2010). However, TERT proteins have an insertion within the palm and fingers domains, a large N-terminal extension, and a C-terminal extension and provide a constant template for reverse transcription through its interaction with TR, with the unique ability for repeat addition processivity (Fig. 12.1d) (Cristofari and Lingner 2003; Lue et al. 2003). Inactivating telomerase in cancer cells could be an effective strategy to limit the extent to which they can divide (Berletch et al. 2008).

There have been several strategies to target telomerases for cancer therapy, including gene therapy, immunotherapy, small molecule inhibitors, oligonucleotide inhibitors, and phytochemicals (Jager and Walter 2016). One example is BIBR1532, a small molecule inhibitor that binds to the FVYL motif of TERT thumb domain (Fig. 12.1d) (Bryan et al. 2015). This inhibitor prevents catalytic activity from occurring; however, it has not shown much efficacy in clinical trials. On the other hand, an oligonucleotide-based lipid conjugate, imetelstat, targets the RNA sequence of telomerase competing with telomere binding (Asai et al. 2003). Imetelstat has undergone clinical trials in patients with hematologic cancers and suffers from dose limitations due to adverse effects (Baerlocher et al. 2015a, b; Tefferi et al. 2015).

Drug discovery targeting telomerase activity has been hampered by the lack of high-resolution information on the multicomponent ribonucleoprotein. Crystal structures of TERT by itself (Gillis et al. 2008), the isolated thumb (Hoffman et al. 2017) and RNA-binding (Harkisheimer et al. 2013) domains, the N-terminal domain (Jacobs et al. 2006), and the C-terminal domain in complex with stem IV of TR (Singh et al. 2012) have been published in the last few years. However, only recently has the architecture of the ribonucleoprotein complex been elucidated by cryo-EM,

allowing visualization of how the pieces fit together (Nguyen et al. 2018). The structure shows that the TR bridges TERT to various other proteins involved in telomerase activity and regulation, with a DNA segment hybridized to the template RNA segment. In the future, this knowledge is expected to facilitate drug discovery based on structural biology information and associated mechanisms.

12.6 Promoting Angiogenesis

As cancer cells proliferate, they reach a critical mass that blocks the formation of new cells due to the lack of oxygen. However, tumor cells overcome this limitation by forming new blood vessels via a process known as angiogenesis (Rajabi and Mousa 2017). This allows tumor cells to continue growing by receiving oxygen and nutrients in their site of origin and to co-opt new tissues as they travel to different organs. The low level of oxygen is referred to as hypoxia, which in cancer cells promotes the expression of vascular endothelial growth factor (VEGF) to stimulate angiogenesis. VEGF regulates angiogenesis through various signaling pathways, and its receptor has been a target for cancer therapeutics for some time (Meadows and Hurwitz 2012). The VEGF family consists of seven members: VEGF-A, VEGF-B, VEFG-C, VEGF-D, VEGF-E, VEGF-F, and placental growth factor (PIGF) (Hoeben et al. 2004). VEGF functions as an antiparallel dimer composed of four central β-sheets and a conserved cysteine knot motif that covalently links each monomer via multiple disulfide bonds: Cys51-Cys60, Cys57-Cys102, Cys61-Cys104, and Cys26-Cys68 (Fig. 12.5) (Muller et al. 1997). The function of VEGF is mediated through binding VEGF receptors: VEGFR1, VEGFR2, and VEGFR3. The structure of each VEGF receptor contains seven immunoglobulin-like domains, a single transmembrane segment, a juxtamembrane segment, an intracellular

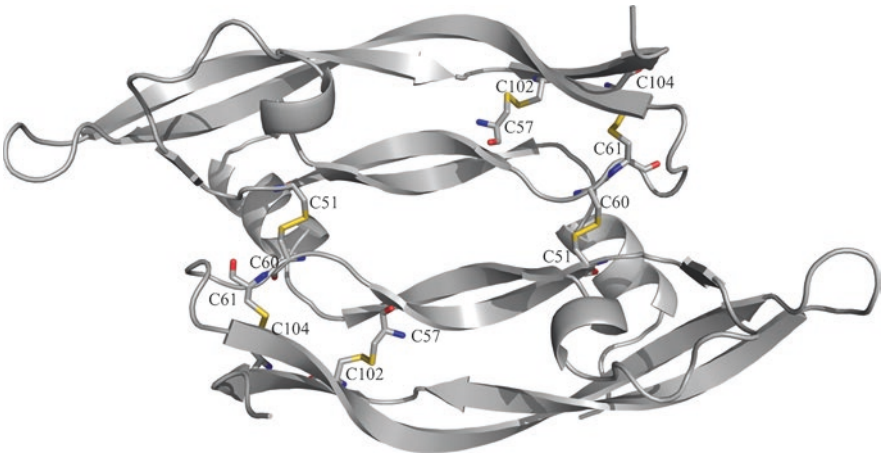

Fig. 12.5 Cysteine knot motif. The three cysteine knots of VEGF are highlighted showing the disulfide bonds in yellow

protein-tyrosine kinase domain, and a carboxyterminal tail (Roskoski 2008). VEGF binds to the second immunoglobulin-like domain (IgG) of VEGFR1 via hydrophobic interactions (Wiesmann et al. 1997), whereas it binds the second and third IgG-like domains of VEGFR2, which is more commonly involved in angiogenic signaling (Fuh et al. 1998). VEGFR2 undergoes activation via *trans* mechanism where VEGF binding induces dimerization resulting in autophosphorylation of the kinase domain (Fig. 12.1e) (Parast et al. 1998).

The interaction of VEGFR2 and VEGF is an appealing target to inhibit angiogenesis (Welti et al. 2013). There have been two major strategies to target the VEGF-VEGFR2 interaction: monoclonal antibodies and small molecule inhibitors. Bevacizumab, a monoclonal antibody, acts as a competitive inhibitor of VEGFR1 and VEGFR2 to disrupt VEGF binding (Welti et al. 2013; von Minckwitz et al. 2012). The crystal structure of VEGF in complex with bevacizumab shows that the VEGF/Fab interaction depends on a small number of residues that confer high affinity residing in a continuous segment of VEGF, different from the multi-segmented interface that interacts with VEGFR2 (Muller et al. 1998). Another inhibition strategy is illustrated by aflibercept, which is a fusion protein of the immunoglobulin domain of VEGF to the Fc region of an immunoglobulin antibody that acts as a decoy receptor with a high affinity for VEGF ligands (Holash et al. 2002). This depletes available VEGF in the cell, therefore leaving VEGFR without a ligand and inactive. Two other antiangiogenic inhibitors that have been approved are sorafenib and sunitinib, selective tyrosine kinase inhibitors that bind to the ATP-binding pocket of VEGFR to prevent ligand binding (Fig. 12.1e) (McTigue et al. 2012). Over the years, many angiogenesis inhibitors have made it to the clinic, but as with inhibition of other cancer traits, these inhibitors are not effective as single drugs due to the plasticity and adaptability of cancer cells: resistance to the drug is rapidly achieved (Torok et al. 2017). It is now clear that there is no single trait that can be targeted alone to cure cancer at advanced stages and that the greatest success is likely to focus on patient-specific approaches to targeting cancer with the right combination of drug cocktails based on structural knowledge of proteins in diverse signaling pathways (Nussinov et al. 2013).

12.7 Activating Invasion and Metastasis

Metastatic cancer is the result of a complicated series of events referred to as the invasion-metastasis cascade resulting in colonization of the primary tumor in distant organ tissues (Lambert et al. 2017). Due to the genetic similarity between the primary tumor and metastatic colonies, it is likely that this occurs at a late stage in the evolution of the cancer cells, such that the mutations associated with the hallmarks of cancer are well established. At this point, the primary tumor has become highly heterogeneous due to rapid mutation and selected evolution in an environment of inflammation and genomic instability. In addition to traits that avoid the cell's defenses, the cancer cells have co-opted cellular metabolism and the immune system to its own benefit. Cells capable of leaving the primary tumor

have undergone epithelial-mesenchymal transition (EMT), acquiring stem cell properties that give it the plasticity and adaptability necessary for successfully establishing colonies in microenvironments distinct from that of the primary tumor cells. Tumor cells eventually leave the primary tumor through intravasation into the circulatory system, where they meet a hostile environment due to stress and the immune system (Headley et al. 2016). There, the circulating tumor cells (CTC) bind to platelets that cover its surface, produce bioactive molecules that aid in the metastatic process (Franco et al. 2015), confer protection against the immune system (Kopp et al. 2009), and sustain progress toward EMT (Lambert et al. 2017). Signaling resulting from tumor cell interactions in the blood promotes neutrophils to secrete matrix metalloproteinases that help with extravasation. Although cells are likely to land in a variety of organ tissues, each cancer type has a propensity to adapt in specific tissues where they can eventually thrive. Thus, breast cancer cells metastasize to bone and brain, while colon cancer often spreads to the liver.

Treatments of metastatic cancer to date have relied heavily on the same approaches and drugs that are used to treat primary tumors (Lambert et al. 2017). Unfortunately, these therapies are not effective against cancer stem cells (CSC) that have undergone EMT, which become highly resistant to treatment (Shibue and Weinberg 2017). One hopeful approach is to target the hepatocyte growth factor (HGF) interaction with the receptor tyrosine kinase c-MET (mesenchymal-epithelial transition factor), which promotes activation of the EMT program, cell motility, and invasion (Jiang et al. 2001). Similar to other receptor tyrosine kinases, the structure of c-Met is composed of a disulfide-linked dimer of an extracellular ligand-binding domain, a membrane spanning segment, a juxtamembrane domain, a catalytic domain, and a C-terminal docking site (Fig. 12.1f) (Ponzetto et al. 1994; Birchmeier et al. 2003). Upon HGF binding to c-Met, the receptor undergoes dimerization and autophosphorylation of two tyrosine residues that are involved in recruitment of downstream molecules (Ponzetto et al. 1994; Stamos et al. 2004). This leads to the activation of various signaling pathways such as MAPK/ERK, PI3K/Akt, STAT, and NF-kB (Trusolino et al. 2010).

The main strategies for targeting c-Met include antagonists for ligand/receptor interactions and the inhibition of kinase activity (Eder et al. 2009). Several inhibitors of c-Met are tyrosine kinase inhibitors designed to block binding of ATP (Goyal et al. 2013; Cui et al. 2011; Qi et al. 2015). Another approach is to target c-Met by miRNAs (Karagonlar et al. 2015). More recently, a non-ATP competitive inhibitor, tivantinib (Fig. 12.1e), has been developed that targets the inactive conformation of c-Met (Munshi et al. 2010). Tivantinib binding causes significant conformational rearrangement of the conserved DFG motif that prevents autophosphorylation, thus stabilizing an inactive conformation (Eathiraj et al. 2011). Tivantinib also binds tubulin, providing an additional benefit in hampering cancer cell growth (Wang et al. 2016; Basilico et al. 2013). As with other kinase inhibitors, tivantinib has failed clinical trials due to the high plasticity and adaptability of cancer cells at the metastatic stage (Rimassa et al. 2018).

12.8 Conclusions

In the past 30 years, there has been remarkable progress in our understanding of the mechanisms through which cancer cells evolve and thrive in an environment that is normally intrinsically hostile to traits that evade the normal homeostasis of cellular processes. One of the difficulties in effective treatment of cancers is that the mechanisms co-opted by tumor cells are the result of an evolutionary process that takes advantage of innate signaling pathways and resources necessary to sustain normal cells. Thus, at the big picture level, a war against cancer is also a war against a co-opted self. Our strategy thus far has been to target pathways that become hyperactive in cancers, such that inhibition may have maximal effects against the tumor with the least detriment to normal cells. Examples of such strategies include targeting proteins in the proliferative pathways, upstream signaling proteins such as receptor tyrosine kinases and downstream cyclin-dependent kinases; telomerase activity, which is dormant in most adult tissues and hyperactivated in cancers; or attacking the uncontrolled formation of blood vessels and the development of EMT programs usurped by cancer cells. By far, most currently available drugs directed at specific proteins have been those targeting kinases, which are regulators of a large number of signaling pathways. This is exemplified in Fig. 12.1, where four of the six hallmark targets we discussed are kinases. Nevertheless, while still relying on nonspecific cytotoxic chemotherapy agents developed in the 1950s and 1960s, clinical treatment of cancer is moving toward a personalized strategy based on cancer cell genome sequencing and targeting of the hallmark traits. Advanced cancer cells respond with remarkable plasticity to most of our existing warheads, such that with few exceptions, "curing" cancer still relies on finding tumors at early, less aggressive stages. Simultaneous targeting of multiple hallmark traits is the hope for the future, but this comes with significant challenges of overcoming toxicity to normal cells. Thus, drug cocktails in combination with targeted delivery to cancer cells will be a powerful approach. While beyond the scope of this chapter, targeted drug delivery (Bi et al., 2016) is an active and thriving area of research that is becoming part of the arsenal of the war against cancer.

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Applications of NMR in Cancer Research

13

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Abstract

Nuclear magnetic resonance (NMR) spectroscopy has been widely applied to many facets of cancer research. As the name implies, NMR spectroscopy is a technique that utilizes transitions between different states of a nucleus when placed in a magnetic field. NMR has been used for decades to identify and quantify the reactants and products associated with chemical reactions. More recently, it has been used in the field termed metabolomics to identify and quantify the small molecules of complex mixtures and how that molecular repertoire varies under various perturbations to the system being studied. NMR is also used for de novo three-dimensional structure determination of macromolecules including proteins and protein domains as well as to quantify the motions inherent in structures. A great strength of the technique is to characterize the interactions of small molecules with these macromolecules, which can be used for inhibitor discovery and development. These applications have direct relevance to cancer research and are described in this review.

Keywords

Nuclear magnetic resonance (NMR) spectroscopy · Cancer · Metabolomics · Biomolecules · Small molecule inhibitor · Molecular dynamics

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13.1 Introduction

Nuclear magnetic resonance (NMR) spectroscopy plays an important role in many aspects of cancer research. NMR has been applied for routine identification and quantification of the reactants and products associated with chemical reactions for many decades. More recently, it has been used to identify and quantify the small molecule components of complex mixtures, in the field termed metabolomics (Wishart et al. 2016). NMR was also developed extensively in the mid-1980s for de novo three-dimensional structure determination of macromolecules (Wüthrich 1989). NMR is also used for measuring the interactions between macromolecules and small molecules. All of these applications have direct relevance to cancer research and are described in this review. NMR has also been used as a diagnostic tool in medicine in the form of magnetic resonance imaging and has been extensively covered in many excellent publications and is touched upon only briefly here (Lin et al. 2017; Brindle 2012; Plathow and Weber 2008; Momcilovic and Shackelford 2018). Likewise, this review restricts itself to solution-phase NMR and does not highlight the exciting new developments in the NMR of solids (Marassi et al. 1997; Renault et al. 2012) or other interesting applications of NMR in cancer that could not be covered due to space limitations.

13.2 NMR Spectroscopy

As the name implies, nuclear magnetic resonance (NMR) spectroscopy is a technique that utilizes transitions between different states of a nucleus when placed in a magnetic field. All commonly studied nuclei are stable, or nonradioactive. The most used nucleus is ^{1}H, the proton, which is contained in practically all biological substances, has 100% abundance, and provides one of the strongest NMR signals of all nuclei, although with less spectral dispersion than most other nuclei. A second commonly used nucleus is ^{13}C, which is present at 1% natural abundance, with the 99% being ^{12}C, which is NMR inactive. When desired to boost the ^{13}C signal, samples can be prepared with isotopic enrichment to attain nearly 100% abundance of ^{13}C. ^{15}N is also used and has even lower abundance (0.01%), and like ^{13}C, samples can be prepared with isotopic enrichment when needed. A fourth commonly used nucleus is ^{31}P, which is 100% abundant.

A typical one-dimensional NMR spectrum comprises a graph in which each nucleus in the molecule or molecules being studied provides a spectral peak. The height of the peak on the y-axis is directly proportional to the concentration. The frequency of the resonance on the x-axis is expressed, somewhat confusingly, as the parts-per-million (ppm) of the overall magnetic field frequency. This allows spectra that have been collected at different overall magnetic field frequencies on different instruments to be more easily compared. The spectral peak will have an observed peak width, typically reported as the width at half-height and may also show fine structure (or splitting) due to what is called J-coupling.

For identifying and quantifying small molecules, typical concentrations are at least 5 μM in a volume of approximately 0.5 mL (or 2.5 nanomoles of material);

some specialized NMR instrumentation designs such as cryoprobes and small-cell probes can achieve tenfold or more higher sensitivity. For macromolecules such as peptides and proteins, where the end goal is three-dimensional structure determination, concentrations are typically about 1 mM. The most commonly used polar solvent is water (H_2O) with the addition of a deuterated component (usually 5% D_2O) for ensuring magnetic field stability. Many other solvents can be used, such as 100% D_2O that reduces the burden for suppressing the resonance arising from H_2O, and chloroform ($CHCl_3$) that can be used to dissolve nonpolar compounds. Hydrogens can be divided in two classes: non-exchangeable (typically those attached to carbon) and exchangeable (those attached to electronegative atoms, such as oxygen and nitrogen). Therefore, the hydrogens of the H_2O solvent can exchange with polar hydrogens on the molecules of interest. A sample that is dried and constituted in 100% D_2O, only the hydrogens bound to carbon remain if the exchange rate is rapid, which is the case for small molecules, but not necessarily for the interior of proteins due to inaccessibility to the bulk solvent (Englander et al. 1972). For ^{1}H NMR of unlabeled compounds, the sample should not include high concentrations of buffer components with non-exchangeable hydrogens (such as glycerol or tris(hydroxymethyl)aminomethane (TRIS)). NMR of compounds labeled with ^{13}C or ^{15}N has fewer restrictions on additives. A typical NMR buffer for determination of metabolite composition is 50 mM phosphate, pH 7. For studies of proteins and peptides, it is usually desired to observe the hydrogens bound to nitrogen; their exchange with solvent can be reduced by lowering the pH (Englander et al. 1972). Some loss of sensitivity occurs with increasing ionic strengths, although usable data can often be obtained that allows successful analysis in high salt conditions (Freedman et al. 1995).

13.3 NMR-Based Metabolomics

13.3.1 Background

Metabolomics is the identification and quantification of the metabolites in a system. NMR has many advantages for the study of cancer processes as it provides a broad but relatively unbiased assessment. While other methods may be more sensitive, they require standard compounds to be run for each instrumental setup. Information can be gained rapidly using NMR from a sample, and since the technique is nondestructive, the sample can be used for other purposes after its analysis. The sample preparation is minimal, for example, no pretreatment is needed at all for urine and blood/plasma/serum. The detection of metabolites is relatively unbiased in that there is generally no preselection of analytical conditions, but the technique still allows for assessment of metabolites over many orders of magnitude in range of concentration. Finally, NMR is highly reproducible, and the spectrum of a compound obtained in one laboratory can be easily replicated in another given the same sample preparation and NMR settings. This reproducibility makes it specifically ideal for epidemiological studies. While NMR has many advantages, the technique does require more material than many other analytic methods.

NMR-based metabolomics has been used in a variety of cancer settings. The greater proliferative potential of cancer cells, often in a hypoxic environment, requires a different metabolic state than normal tissue. Otto Warburg first described the rapid consumption of glucose and the large production of lactate by tumors, even in the presence of oxygen, which is now known as the "Warburg" effect (Vander Heiden et al. 2009; Warburg et al. 1927). More recently, it is appreciated that particular cancers have particular metabolic vulnerabilities, which has led to unique and specific metabolic approaches to target tumor cells. One example is the systemic delivery of asparaginase to reduce serum asparagine levels to treat acute lymphoblastic leukemia and acute myeloid leukemia (Hill et al. 1967). A second example is an orally available small molecule inhibitor that targets mutated forms of isocitrate dehydrogenase (IDH) that reduces formation of the oncometabolite 2-hydroxyglutarate (2HG). This prevents 2HG-mediated signaling and inhibits cellular proliferation in tumor cells expressing IDH mutations, such as those associated with the majority of low-grade gliomas and secondary glioblastomas and chondrosarcomas (Molenaar et al. 2018).

13.3.2 Metabolic Assessments Using In Vitro Model Systems

NMR has been used extensively to interrogate systems that recapitulate cancer in vitro. Many published studies have focused on alterations in cellular metabolism that occur with a gene mutation or with the administration of an anticancer drug (Pavlova and Thompson 2016; Vander Heiden and DeBerardinis 2017). Two approaches are typically used: the steady-state approach, which assesses the metabolite levels at a given time in the cell, and the flux approach, where cells are incubated with a labeled substrate for a short period of time and the metabolism of that substrate is followed explicitly.

For example, we recently explored how various cancer cell lines increase their metabolism to create the molecular building blocks necessary for proliferative cell growth (Pattni et al. 2017). We supplied A2780, a human ovarian carcinoma cell line, with uniformly $^{13}C_6$-labeled glucose. Under normal growth conditions, the labeled glucose ended up in several amino acids derived from TCA cycle and glycolytic intermediates with some glucose remaining unmetabolized. However, in the presence of NCL-240, an anticancer drug of the PITenin class of PI3K-Akt inhibitors, all the glucose was metabolized, and the label appeared almost exclusively in lactate. These data support the conclusion that NCL-240 caused a disruption in mitochondrial oxidative phosphorylation and upregulated glycolysis. Subsequently, NCL-240 was combined with 2-deoxyglucose (2-DG) in proof-of-concept studies of several cell lines that exhibited enhanced cell death response to administration of the combination of NCL-240 and 2-DG. Compared to control conditions and individual treatments, significant improvements in cytotoxicity were shown in multiple cell lines (Pattni et al. 2017).

As an alternative to examining metabolic changes within cells, one can look at the small molecules that cancer cells consume and secrete to assess how the cells

react and adapt to the conditions and surrounding environment in which they grow. To investigate the effects of different culture media on the morphology and growth patterns of cancer cells in monolayer cell cultures, we recently performed metabolomic assessments of conditioned culture media. Cultures of human basal cell carcinoma (BCC) cells were established by seeding 2.5×10^5 BCC cells (TE 354.T, ATCC Manassas, VA) in two different growth media (DMEM∗ and AmnioMAX). DMEM∗ is ATCC-formulated Dulbecco's Modified Eagle's Medium (DMEM) and is modified to contain 4 mM L-glutamine, 4500 mg/L glucose, and 1 mM sodium pyruvate, supplemented with 10% fetal bovine serum (FBS, HyClone, Thermo Scientific, Rockford, IL), penicillin (100 units/ml), and streptomycin (100 μg/ml) (Gibco® Life Technologies) according to manufacturer protocol. AmnioMAX is Gibco AmnioMAX™ C-100 Complete Medium that contains FBS, L-glutamine, and gentamicin to maximize cell attachment and growth (Fisher Scientific, USA). BCC cell cultures were maintained in T-25 flasks in a humidified incubator at 37 °C with 5% CO_2. Sub-confluent cultures were trypsinized, and cells were reseeded either in the same growth media prior to trypsinization or were exposed to the other type of growth media. Cells were imaged with an inverted Axiovert 40CFL Zeiss microscope (Gottingen, Germany) using Infinity Software and a Lumenera camera (Lumenera Corp., Ottawa, Canada) (Fig. 13.1), while samples of conditioned media

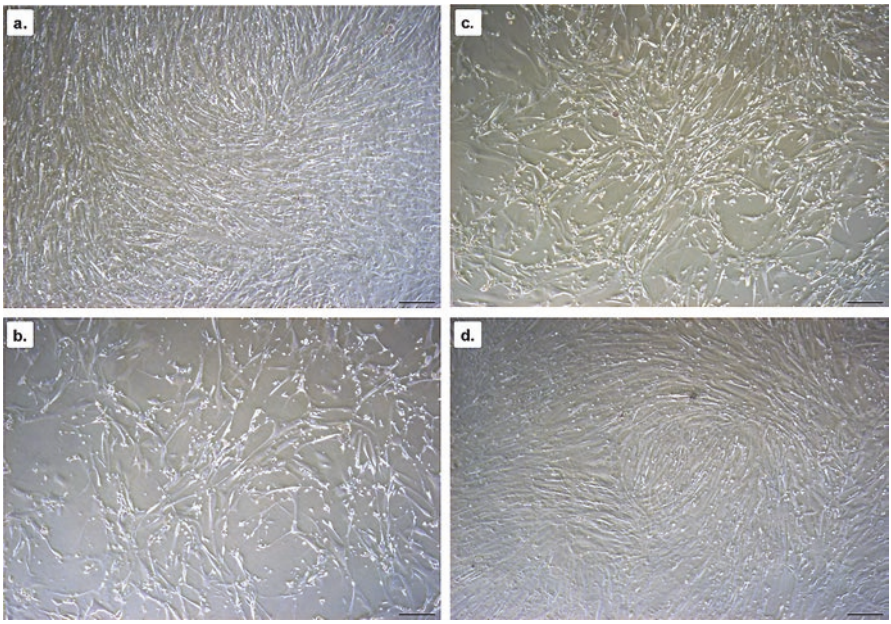

Fig. 13.1 Effects of growth culture media on the morphological characteristics of human basal cell carcinoma cell cultures. BCC cell cultures grown in ACC-modified DMEM media showed confluent areas of uniform, elongated cells that grew in curved patterns (**a**), while BCC cultures grown in AmnioMAX media showed scattered areas of elongated cells and abundant rounded cells (**b**). Exposing BCC cultures that grew in ATCC-modified DMEM media to AmnioMAX medium (**c**), and vice versa (**d**), resulted in rapid phenotypic changes that resembled the morphology seen in cultures that continuously grew in the same media type. Scale bar = 100.0 microns

from the cultures at days 5, 10, and 15 were also harvested. Aliquots of 0.5 mL of media were buffered to 50 mM phosphate, pH 7.2 for ^{1}H NMR spectroscopy at 600 MHz. 1-D spectra were acquired and processed according to published protocols (Weljie et al. 2006).

Cultures of BCC cells that continuously grew in DMEM∗ media demonstrated confluent areas of elongated cells with fibroblast-like morphology that grew in compact wave patterns (Fig. 13.1a). In contrast, BCC cultures that continuously grew in AmnioMAX media exhibited areas of elongated cells that grew in less compact curved patterns along with the appearance of numerous small rounded cells that closely surrounded them (Fig. 13.1b). The morphology of BCC cells that were grown in DMEM∗ media and switched to AmnioMAX medium changed rapidly, within 24–48 h, and was similar to that seen in cultures that continuously grew in that growth medium (Fig. 13.1c). Similarly, the morphology of BCC cells that were grown in AmnioMAX medium and switched to DMEM∗ media was comparable to that seen in cultures that continuously grew in that growth medium (Fig. 13.1d). These results demonstrated the rapid morphologic plasticity of human BCC cancer cells in response to their surrounding media when grown as cultures in vitro.

In order to understand the difference in growth patterns and morphology of the BCC cell cultures, we performed metabolomic assessment of the media in which they were grown. We assessed metabolic profiles of fresh DMEM∗ and AmnioMAX media as well as conditioned media from BCC cell cultures that either grew continuously in each one of these media types or were switched from one growth media to the other. Here, we discuss the levels of two representative metabolites (pyruvate and arginine) out of the few dozen quantified metabolites. Pyruvate is one of the three main oxidative fuels of mitochondria, together with glutamine and fatty acids. DMEM∗ media, which is recommended for the growth of human BCC cells, contained higher level of pyruvate than AmnioMAX media (Fig. 13.2a, b, white bars). BCC cell cultures consumed pyruvate when continuously grown in DMEM∗ media (Fig. 13.2a, gray bars) but secreted this metabolite when switched to grow in AmnioMAX media (Fig. 13.2b, gray bars). Similarly, BCC cell cultures secreted pyruvate when continuously grown in AmnioMAX media (b, black bars) but consumed it when switched to continue their growth in DMEM∗ media (a, black bars). The metabolite arginine is an amino acid that is used in protein biosynthesis and is important for multiple cellular and tissue functions, such as cell division and wound healing. DMEM∗ media contained less arginine than AmnioMAX media (Fig. 13.2c, d, white bars). BCC cells secreted arginine when continuously grown in DMEM∗ media (Fig. 13.2c, gray bars) but consumed this metabolite when switched to grow in AmnioMAX media (Fig. 13.2d, gray bars). These cells consumed arginine when continuously grown in AmnioMAX media (Fig. 13.2d, black bars) but secreted it when switched to grow in DMEM∗ media (Fig. 13.2c, black bars). These results demonstrated the ability of human BCC cells to switch from consumption of specific metabolites from the growth media to secretion of these metabolites into the media, which indicated the rapid adaptive capabilities of these cells to their surrounding environment. This adaptation is likely to be associated with a phenotypic

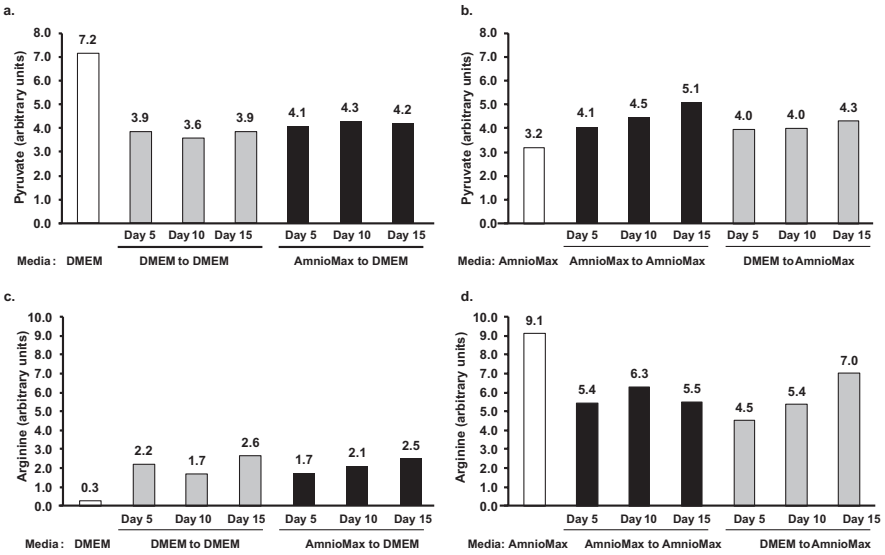

Fig. 13.2 Effects of human basal cell carcinoma cell cultures on growth culture media. ATCC-modified DMEM media contains high level of pyruvate (**a**) and low level of arginine (**c**), whereas AmnioMAX media contains a lower level of pyruvate (**b**) and a higher level of arginine (**d**). When continuously grown in ATCC-modified DMEM media, BCC cells consumed pyruvate and secreted arginine (**a**, **c**, gray bars, respectively). Conversely, when continuously grown in AmnioMAX media, these cells secreted pyruvate and consumed arginine (**b**, **d**, black bars, respectively). When the BCC cell cells were initially grown in one media type and thereafter switched to grow in the other media type, the same consumption and secretion patterns were identified. Metabolite levels are expressed as normalized, arbitrary units

change and the activation of different cellular metabolic pathways that, in turn, can potentially become new biomarkers for disease or targets for metabolic-based cancer therapies.

13.3.3 Metabolic Assessment In Vivo

The state of a tumor mass can also be assessed by taking a biopsy of the tumor or the bodily fluids with which the tumor interacts (namely, blood and urine). For example, recently we described a unique patient-derived xenograft (PDX) and cell culture model of succinate dehydrogenase-deficient gastrointestinal stromal tumor (SDH-deficient GIST) (Powers et al. 2018). This model is not only important for what it may show specifically about this rare specific tumor type but also other types of SDH-deficient tumors. This model is the first from an SDHB-mutated human tumor that can be experimentally manipulated to study the effects of oxygen deprivation and novel treatment regimens. The data support a hypothesis that tumor growth requires the positive protective effects of hypoxic signaling without the deleterious effects of oxidative stress. In these experiments, the tumor was

extracted using water/methanol/chloroform mixture; then, the water/methanol extract was dried and submitted for NMR analysis. The steady-state metabolic assessment confirmed that succinate levels were high, exceeding 10% of the total of all observed metabolites, whereas a non-SDH-deficient control tumor had succinate levels of about 0.5%. Thirty minutes prior to sacrifice, mice had been injected intraperitoneally with 100% uniformly $^{13}C_6$-labeled glucose to allow us to follow the fate of the ^{13}C-label. In SDH-deficient mice, ^{13}C-glucose label was found in glutamate as well as other metabolites. The glutamate was labeled equally as [$^{13}C_2$-4,5]Glu and [$^{13}C_2$-2,3]Glu isoptomers indicating equivalent activities of the pyruvate dehydrogenase and the anaplerotic pyruvate carboxylase pathways (Bruntz et al. 2017; Lussey-Lepoutre et al. 2015). There was also significant incorporation of label into succinate. In contrast, the ratio of [$^{13}C_2$-4,5]Glu to [$^{13}C_2$-2,3]Glu isoptomers in the non-SDH-deficient xenograft was about 6:1, without labeling into succinate. These findings are consistent with the increase in pyruvate carboxylase activity as previously reported in a different SDH-deficient cell line (Lussey-Lepoutre et al. 2015).

While tumors can be extracted to yield solutions that can be assessed in a conventional NMR spectrometry, one can add HR-MAS functionality to some NMR systems to allow measurements on intact tissue specimens (Somashekar et al. 2011; Li et al. 2011; Tessem et al. 2008; MacKinnon et al. 2012; Sjobakk et al. 2013; Choi et al. 2012; Mun et al. 2016; Wilson et al. 2009). By spinning the sample rapidly at a particular "magic" angle, narrow peaks can be obtained to allow metabolomic measurements. The advantage of these high-resolution magic-angle spinning (HR-MAS) experiment is that the sample requires only minimal processing and can be used then for other purposes after its analysis. On the other hand, extraction using methanol and chloroform renders the sample relatively enzymatically inert and thus more stable. In addition, while HR-MAS spectra are very good, they still have poorer resolution than conventional solution phase NMR for which instrumentation is more readily available.

An alternative to assessing the tumor mass directly is to measure samples of blood and or urine from the host organism that report on the environment of the tumor (Stretch et al. 2012; Kind et al. 2007; El-Sayed et al. 2002; Mathe et al. 2014; Johnson et al. 2012; Yang et al. 2018; Ludwig et al. 2009; Tenori et al. 2012; Mayers et al. 2014; Louis et al. 2016). While not as direct as characterizing the tumor itself, both blood and urine can be obtained either in a minimally invasive manner (venipuncture in the case of blood) or noninvasively in the case of urine. In this case, because the sample size is not usually a limiting factor, the technique lends itself sufficient sample to allow for a combination of analytic methods, such as mass spectrometry to complement the NMR studies that provides a much deeper understanding of the metabolism of the tumor than the separate techniques (Rowan et al. 2017; Yanshole et al. 2014; Bingol and Bruschweiler 2015).

A new methodology called hyperpolarization has much promise to reduce the insensitivity associated with ^{13}C NMR. Such a method increases the signal-to-noise ratio by factors of at least 100 or more. Although hyperpolarized nuclei have a short lifetime, they have been used in demanding in vivo applications (Nelson et al. 2013)

as well as other study settings (Momcilovic and Shackelford 2018; Comment and Merritt 2014; Jeschke and Frydman 2016).

13.4 Biomolecular Structure and Function

13.4.1 Overview of Three-Dimensional Structure Determination Using NMR Methods

NMR can be used in addition to X-ray crystallography and cryoelectron microscopy, to determine the three-dimensional structure of proteins that relate to cancer function. The advantages of NMR are that no crystals need to be obtained and that the sample remains in the solution phase. The exact NMR methodology depends on the size of the protein to be studied. Here, the structure determination of a lipidated peptide, termed a pepducin, is described in detail. Structure determination of other proteins relevant to cancer is described in subsequent sections.

13.4.2 Application to Unlabeled Peptides

Pepducins are highly stable lipidated intracellular loop segments from GPCRs that are specific for their cognate receptor (Covic et al. 2002a, b). The rapid and efficient cell membrane flipping and membrane tethering of pepducins (Covic et al. 2002b; Wielders et al. 2007; Miller et al. 2009; Janz et al. 2011; Tsuji et al. 2013) make them well suited for interrogating the roles of intracellular regions of receptors in G-protein coupling and signaling. Libraries of pepducins can be readily constructed to target a GPCR of interest based on the sequences of the intracellular loops and tested for activity by high-throughput screening (Tchernychev et al. 2010). This approach has proven useful in delineating the function of specific GPCRs in cancer (Boire et al. 2005; Yang et al. 2009; Cisowski et al. 2011; O'Callaghan et al. 2012), cardiovascular (Covic et al. 2002b; Kuliopulos and Covic 2003; Leger et al. 2006; Trivedi et al. 2009; Tressel et al. 2011a) and inflammatory (Kaneider et al. 2005, 2007; Sevigny et al. 2011; Tressel et al. 2011b) diseases. Although pepducins have entered human clinical trials (Zhang et al. 2012a; Gurbel et al. 2016), their mechanism of action is still largely unknown. As described below, we determined the structure of a PAR1 antagonist pepducin, using NMR methods. Together with the published crystal structure of PAR1 that lacked density for the C-terminus (Zhang et al. 2012b), the NMR data provide a more complete picture of PAR1 function. Unlike larger proteins, NMR methods on peptides do not require isotopic labeling with ^{15}N or ^{13}C.

A peptide of 13 amino acid residues was chosen to represent the presumed helical C-terminal fourth intracellular loop of PAR1 and termed P1i4H8. This pepducin was synthesized with a palmitate tail on the N-terminus and NH_2 at the C-terminus and has the primary sequence ASSGSERYVYSIL (corresponding to residues 374–386 in PAR1, and renumbered here as residues 2–14, with residue 1 designated as

palmitate). P1i4H8 was previously shown to inhibit Gq-PLC-β signaling, whereas a mutated pepducin predicted to have a distorted helix had no effect (Swift et al. 2006). The HPLC-purified peptide was dissolved to final concentration of about 1 mM in a buffer comprising 91 mM perdeuterated dodecylphosphocholine, 9 mM deuterated acetic acid at pH 4.7, 50 μM DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) NMR standard, 91% H_2O, and 9% D_2O. NMR spectra were collected 25 °C on a Bruker Avance 600 spectrometer (Zhang et al. 2015) and included two-dimensional nuclear Overhauser effect correlation spectroscopy (NOESY, mixing time of 100 msec) and total correlation spectroscopy (TOCSY, mixing time of 40 msec). Proton assignments were made according to standard homonuclear methods (Wüthrich 1986, 1990) using DSS as an internal standard calibrated to 0.0 ppm. When comparing the experimental chemical shift values against the corresponding values for unstructured peptides, the alpha proton of almost all residues showed upfield shifts (an average difference of −0.31 ppm) from random coil chemical shift values, suggesting a helical secondary structure (Wishart and Sykes 1994). The one outlier was the third serine in the sequence that indicated a point of flexibility that strays from an ideal alpha helix. The NOEs characteristic of helical structure were then tabulated (Fig. 13.3). These include NOEs between amide protons of a given residue (i) to the next residue in the sequence (i + 1) and to the residue after that (+2), alpha protons of residue i to amide protons of residues i + 2 and i + 3, and alpha protons of residue i to beta protons of residue i + 3 (Fig. 13.3). Consistent with the number of NOEs per residue and variation from random coil chemical shifts, the peptide is predominantly alpha helical – with greatest confidence in the region from residue 8 to residue 13.

The NOE data were then converted into distance restraints following previously described procedures (Hyberts et al. 1992). Non-stereospecifically assigned protons were given distance corrections of 1.0 Å for methylene proton ambiguities and 2.0 Å for the ring protons of tyrosine. If methylene protons pairs produced NOEs with another proton, the difference in these distances was added to upper and lower distance constraint boundaries. Proton and carbon chemical shifts and distance restraints were used in CNSsolve version 1.3 to generate structures. Ten initial structures were generated, and NOE violations were identified using CcpNmr

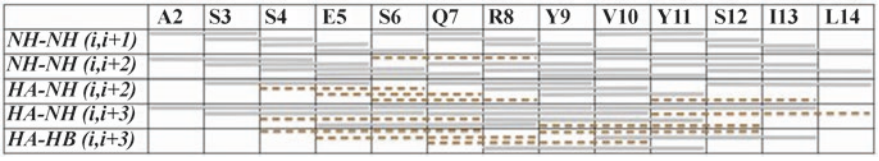

Fig. 13.3 Inter-residue NOEs indicating the extent of alpha helical secondary structure within the i4H8 pepducin. Solid, gray lines demonstrate a confident NOE from the NOESY spectrum, and brown dashed lines represent possible NOEs that may be obstructed by resolution or overlapping peaks

Analysis (Skinner et al. 2015). The structures were refined, guided by violation recognition and severity. Iterations of refinement were completed by adding half of the mean violation per restraint to the upper and lower bound of the given restraint until there were no significant violations (>0.1 Å). After generation of the finalized distance constraint list, 30 refined structures and an average of 30 structures were generated with CNSsolve. A Ramachandran plot of the average structure from the ensemble of 30 accepted models for P1i4H8 was developed using PROCHECK-NMR through the PSVS validation server (Laskowski et al. 1996). All residues are in the alpha helical region of the plot, with 64% in most favored regions and 36% in additional allowed regions. Refined structures were superimposed upon one another with a root-mean-square deviation (RMSD) of 0.3 Å, and an average structure was calculated (Fig. 13.4). The crystal structure of a homolog, PAR2, has been determined that includes the eighth helix (Cheng et al. 2017). Although having only 15% amino acid identity in this region, the P1i4H8 pepducin could be overlaid with the corresponding region in PAR2, with a root-mean-square deviation of only 0.8 Å in backbone atoms. This overlay allows us to create a model of PAR1 that includes the eighth helix. E377 of PAR1 was about 7 Å away from the side chain of K135 and, with some minor rearrangements, could make an electrostatic bond earlier posited based on mutational analysis (Swift et al. 2006). Q379 in this model is in close contact with Y372 of the seventh transmembrane helix (about 3 Å) and provides further evidence for a 7-1-8 model of PAR1 mechanism (Swift et al. 2006; Zhang et al. 2015).

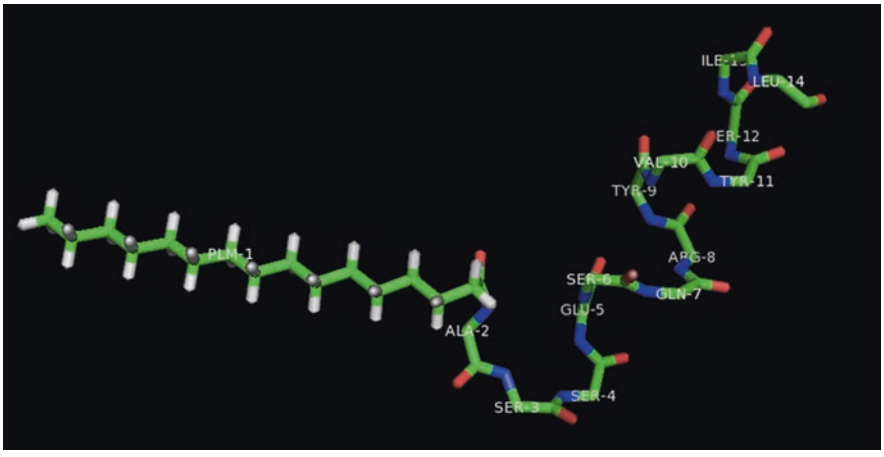

Fig. 13.4 Depiction of the P1i4H8 pepducin structure. The structure was derived from 340 distance restraints determined by NMR spectroscopy. The palmitate group (PLM-1) was forced to be linear using conformational restraints. The average of an ensemble of structure (root-mean-square deviation of 0.3 Å) was calculated and minimized, and only backbone atoms are shown for clarity

13.4.3 Applications to Oncoproteins and the Ras Family of Proteins

Another application of NMR is structure determination of small protein domains which have tumor-promoting capabilities. Human papillomavirus (HPV) is a causative agent of cervical and anogenital cancers. The virus encodes a small number of proteins, namely, the E1, E2, E6 and E7 proteins, which allow to coercion of the host cell to allow viral propagation. The oncogenic E6 protein from high-risk HPV types 16 and 18 contains two zinc-binding domains. E6 initiates cell transformation through formation of a complex with E6AP, which binds p53 and stimulates its degradation. There were few biophysical studies due to difficulty in preparation of soluble protein; we and others have described the preparation of soluble E6 constructs such as the two individual zinc-binding domains of E6 (Liu et al. 2007, 2009; Chi et al. 2011; Sidi et al. 2011; Nomine et al. 2005) that eventually allowed for three-dimensional structure determination of the full oncoprotein using NMR (Zanier et al. 2012) and crystallography (Martinez-Zapien et al. 2016) methods.

The three-dimensional structure for the portion of E6AP that binds E6 has been shown to be helical (Liu et al. 2004; Be et al. 2001). Alanine-scanning mutagenesis suggested the most critical residues of E6AP for binding E6, which is supported by the crystal structure of their complex (Martinez-Zapien et al. 2016). The placements of the atoms in E6AP were subsequently used for selecting small molecular weight compounds that inhibit E6 (Baleja et al. 2006; Cherry et al. 2013).

We also used NMR to determine the structure of the complex of a second interacting protein partner (PDZ2) bound to a peptide derived from the HPV-18 E6 protein (Liu et al. 2007). The resultant structures had backbone root-mean-square deviations of less than 0.5 Å, suggesting that they were of high quality. The structure showed a novel mode of interaction in which the C-terminal six residues of HPV-18 E6 protein are contacted by the PDZ2 domain, in contrast to the four residues typically used by similar class (class I) PDZ domains. Comparison of this structure to previously determined X-ray structures of apo PDZ2 and PDZ2 bound to different peptides allowed a description of conformational changes needed in PDZ2 and in HPV-18 E6 to form a molecular complex.

We and others have also studied the E2 protein of papillomavirus using NMR (Liang et al. 1996; Veeraraghavan et al. 1999; Bose et al. 2007, 2011). The viral E2 protein plays a role in viral DNA replication and in the transactivation of viral oncogenes and thus represents a possible target for therapeutic inhibition for preexisting HPV infections. Dimerization of E2, which takes place via a conserved C-terminal DNA-binding domain (DBD), is critical for function. The conservation of two histidines (H290 and H320) at the dimer interface suggested that they play important roles in protein structure and stability. We determined their pKa values by NMR and mutating these residues to neutral alanine (Bose et al. 2007). We demonstrated that at neutral pH (7.5), the wild-type DBD was more stable than either of the two histidine mutants. However, at acidic pH (4.5), the order of stability changed where the H290A mutant was most stable. Although H290 is important for pH-induced protein instability, it was not critical for dimerization or folding at lower pH. We also

determine pKa values for the other, solvent-exposed histidine residues, which demonstrate that the surface properties of the protein change with pH. Moreover, identification of residues crucial for E2 stability helped in the design of a modified protein with desirable characteristics as a viral inhibitor (Bose et al. 2011).

The Ras superfamily of G-proteins are monomeric proteins of approximately 21 kDa that bind GTP and act as a molecular switch to regulate cellular processes including cancer (Tetlow and Tamanoi 2013). The structures of Ras proteins and their interacting partners have been well characterized using NMR methods. Multiple conformational states have been elucidated that are relevant to function (Parker et al. 2018; Schopel et al. 2017). Because Ras is either overexpressed or mutated to an active conformation in a great variety of cancers; much excitement is in the field over the possibility of developing specific small molecule inhibitors for Ras (Shima et al. 2015).

13.4.4 Assessment of Binding

Above we illustrated that NMR can be used to determine the pKa values for ionizable amino acid residues, which in reality is assessing the binding of protons to individual sites within a protein. NMR can also be used to assess the binding of almost any molecule to a protein. Here, the usual methodology is to express the protein in a medium containing ^{15}N as a sole source of nitrogen and then to collect ^{15}N-^{1}H two-dimensional correlation (HSQC) spectra as a function of the added ligand (Fig. 13.5). Typically, each amino acid residue except proline, shows a single

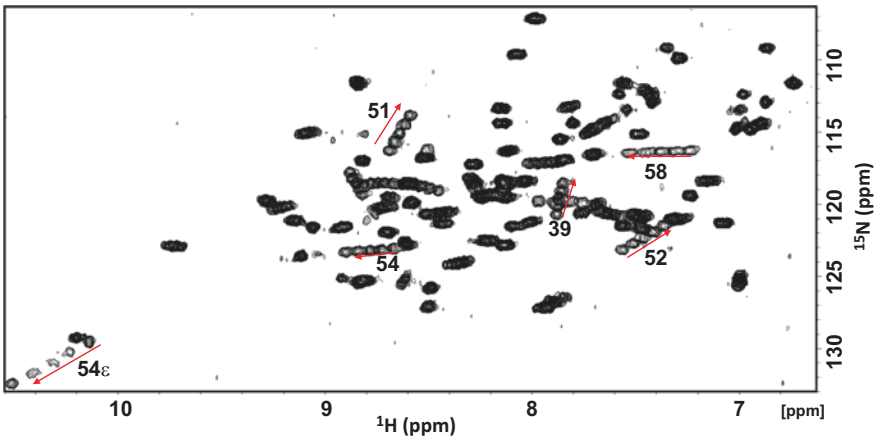

Fig. 13.5 Superimposed ^{15}N-^{1}H HSQC spectra of the Reps1 EH domain with 0, 0.25, 0.5, 1, and 2 equivalents of an NPF-containing peptide derived the Rab/Rip protein. The 300 MHz spectra were collected on samples containing approximately 0.4 mM EH domain. As is typical, the two-dimensional peaks are represented as contour plots, with higher peaks showing more contours than weak peaks. Peaks that shift significantly (with minimal spectral overlap) on addition of peptide are marked with arrows and residue numbers. Four other peaks that changed are not marked for clarity

peak in the two-dimensional spectrum corresponding the NH unit of the peptide backbone. In addition, the ε NH unit of the tryptophan side chain and the NH_2 units of the side chains of asparagine and glutamine are also observable at near-neutral pH. We illustrate the utility of this approach with the Eps15 homology (EH) domain, a conserved sequence of about 100 amino acid residues first described in the human protein Eps15, which is found 19 times in 11 human proteins (Confalonieri and Di Fiore 2002; Oosterhoff et al. 2003). EH domain-containing proteins are fundamental in directing the movement of proteins and other molecules to and from the cell surface. EH domain-containing proteins interact with proteins that have a sequence of three amino acids (Asn-Pro-Phe; NPF). There are numerous potential binding proteins containing multiple NPF motifs. While some selection is mediated by tissue type, temporal expression, and intracellular localization, other regulation is likely to be via direct contact to residues adjacent to NPF or via the way in which the conformation of NPF is influenced by the neighboring residues. The domain from Reps1 is of interest to cancer because it was identified as a binding partner of RalBP1, which is a downstream target protein of the Ral GTPase and a protein implicated in many cancer pathways (Yamaguchi et al. 1997; Gentry et al. 2014). The portion of the Reps1 (residues 227–318) representing the EH domain (renumbered as residues 1–97) was prepared as previously described (Kim et al. 2000, 2001). Figure 13.5 represents the HSQC spectrum for the EH domain as different amounts of the peptide PTGSSSTNPFL, representing an NPF-containing binding partner Rab/Rip (Yamabhai et al. 1998; Bogerd et al. 1995; Fritz et al. 1995), were titrated into the sample. While many amino acid residues showed no significant change in their NH resonances, a group of about ten amino acid residues showed significant changes. These can be mapped onto the three-dimensional structure of the domain and show the same contiguous surface as previously observed for a similar peptide (Kim et al. 2001). The chemical shift changes can be interpreted with respect to the EH domain and peptide domain concentrations to calculate the equilibrium dissociation constant (here $K_d = 60 \pm 15$ μM). Inspection of the ε NH for tryptophan 54 shows broadening and reduced intensity of the resonance at intermediate stoichiometries of peptide to EH domain (Fig. 13.5). The presence of sharp peaks for the free and bound states of the domain suggests that the peptide bound with intermediate exchange, which allows for calculation of the off rate (approximately 500 s^{-1} in this case) (Kim et al. 2001).

While the chemical shift changes of NMR are the mainstay method for characterizing molecular interactions, other techniques are also used, such as DOSY, saturation-transfer difference spectroscopy, and WaterLOGSY, and have been reviewed elsewhere (Sugiki et al. 2018).

13.4.5 BCL and Small Molecule Inhibitor Discovery

Fesik and colleagues have used an innovative approach called structure-activity relationships by NMR (SAR by NMR) to select and optimize small molecule inhibitors (Shuker et al. 1996; Hajduk et al. 1999; Hajduk 2006). These fragment-based approaches are now commonly used for inhibitor modification. In short, one uses the HSQC spectrum of a protein to find chemical fragments that bind to adjacent

sites. These chemical fragments are then coupled to create higher affinity ligands. For example, the Bcl-2 family of proteins regulates apoptosis, and overexpression of the anti-apoptotic members of this family, such as Bcl-2 and Bcl-xL, improves the resistance of cancer cells to chemotherapeutic agents. To identify initial ligands that could serve as starting point for subsequent optimization, human Bcl-2 was screened against a library of 17,000 compounds with an average molecular weight of 225 using ^{1}H-^{13}C HSQC spectra (Petros et al. 2010). A diphenylmethane was found to bind Bcl-2 with a Kd of 20 μM. Previous structural data suggested that biaryl acid ligands would bind adjacent to the diphenylmethane (Petros et al. 2006). Structure determination of the ternary diphenylmethane/biaryl acid/ BCL-2 protein complex provided important clues as to how to chemically link the two adjacent ligands and yielded compounds with inhibition constants in the low micromolar range. Further optimization resulted in a compound with an inhibitory constant of about 40 nM (Petros et al. 2010). BCL-2 inhibitors are now used clinically to treat multiple hematological and other malignancies (Montero and Letai 2018). The future has many exciting opportunities for the development of inhibitors of protein-protein interfaces, previously thought to be undruggable targets (Sugiki et al. 2018). This protein and the ones described above have quite a small size. NMR applications of inhibitor design appropriate for larger proteins and protein complexes have been recently reviewed (Dias and Ciulli 2014).

13.5 Molecular Dynamics

A unique feature of NMR spectroscopy is the ability to define the dynamics of a macromolecule. One of the earliest experiments was to resolubilize a protein in D_2O and watch the disappearance of NH peaks as the hydrogens exchanged with deuterons (Englander et al. 1972; Mau et al. 1992). Subsequently, the use of NMR has blossomed, with the development of many techniques covering a wide range of motional timescales, which have been extensively reviewed (Ishima and Torchia 2000; Akke 2002; Kay 2005; Eisenmesser et al. 2005; Mittermaier and Kay 2009; Palmer 2015). Such studies have revealed, for example, that residues in the active sites of enzymes have motional characteristics that help drive the catalytic reaction (Kern and Zuiderweg 2003). A new development in the field is the use of dynamic information to help design better inhibitors. Conformational dynamics on a range of timescales are associated with enzyme auto-inhibition and allosteric regulation, substrate binding, enzyme-substrate complex remodeling, and product release, all of which may be exploited to derive better enzyme inhibitors applicable to cancer (Palmer 2015).

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Proteomics for Cancer: Approaches and Challenges

14

Surekha M. Zingde

Abstract

The draft human genome sequence was available in 2003. It is now basic knowledge that there are ~20,300 protein coding genes in the human genome which undergo regulated transcription and posttranscriptional processing to generate several mRNA. These in turn are translated and posttranslationally modified to generate over two million or more protein products which are the actual work horses in the cell. The comprehensive analysis of these proteins, i.e. their identity, characteristics, amino acid variations, location, turnover, proteolytic processing, posttranslational modifications and subunit associations, is the realm of proteomics. This chapter will focus on the major developments in technology which have contributed to the evolution of proteomics and the use of these for understanding cancer biology with the aim to generate data which will translate from the bench to the clinic.

Keywords

Proteomics · Cancer · HUPO · Proteomic tools · Mass spectrometry · Protein arrays · Signalling

14.1 What Is Proteomics?

The sequencing of the human genome brought to attention the need to identify the genes coding for proteins. The central dogma which is well accepted shows that there is regulated expression of the genes at the transcriptional level which leads to

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several mRNAs due to splicing of exons in different ways in different tissues and conditions. Further, mutations and polymorphisms in the genes lead to mRNAs with different sequences. These could be around 200,000 mRNA. The mRNAs have varied half-lives. These mRNAs in turn are translated into proteins with different sequence information referred to as isoforms. The proteins are further posttranslationally modified to yield glycosylated, phosphorylated, acetylated, and many other modifications (dPTM:http://dbptm.mbc.nctu.edu.tw/; Aebersold et al. 2018). The proteins so generated from the same gene either exist as single subunits or have multi-subunits which determine their solubility, activity, location, abundance, association with other proteins, half-life, turnover, dynamics and tissue distribution. The study of all the proteins and their characteristics under variety of conditions and in different tissues is proteomics.

14.2 Human Proteome Organization

The Human Proteome Organization (HUPO) launched the Human Proteome Project (HPP) in 2010 to map the entire human protein set. It has focussed on three major technologies to meet its targets. They are mass spectrometry (MS), antibodies and knowledge base, i.e. bioinformatics (Legrain et al. 2011) Fig. 14.1.

The HPP has two major goals (Omenn and Lane 2016; Omenn 2017; Omenn et al. 2017):

1. The first goal is to complete the protein parts in a stepwise manner, by confidently identifying and characterizing at least one protein product from each

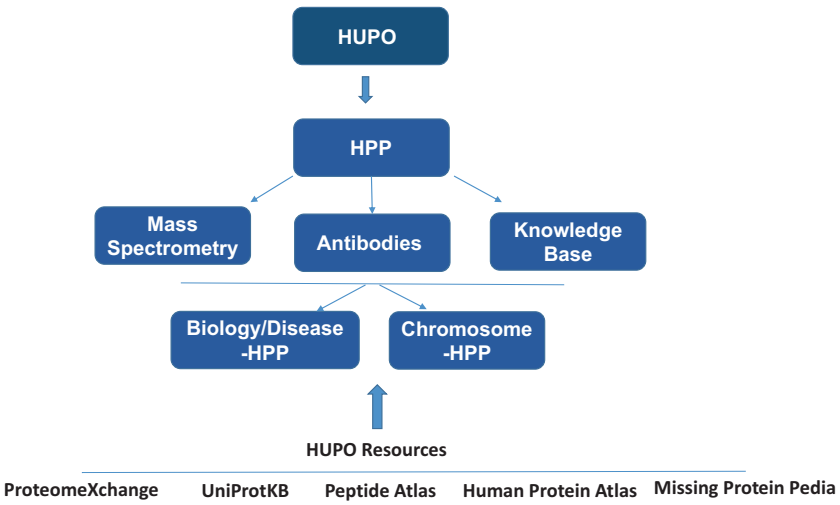

Fig. 14.1 The various components of the Human Proteome Organization (HUPO). *HPP*-Human Proteome Project

protein-coding gene along with the sequence variants, posttranslational modification (PTMs), single amino acid variants (SAAVs), splice isoforms and functions of the proteins.
2. The second goal is to integrate proteomics into the multiomics analysis of biomedical and life sciences research by developing assays, instruments, knowledge bases for identification, quantitation and functional assessment of proteins and proteoforms in diverse biological systems. The overall aim is to provide a platform for addressing disease management.

There are 50 HPP projects involving participation of several countries (Omenn 2017). They are the Biology and Disease HPP (22 projects) (Van Eyk et al. 2016), Chromosome HPP (25 projects) (Paik et al. 2017), Human Proteome Atlas (HPA, http://www.proteinatlas.org) and the Knowledge Resource Pillar of the HPP. The biology and disease B/D-HPP addresses different clinical conditions and has achieved a lot since its beginning in 2012. Today this project has an SRMAtlas with 166 K uniquely mapping proteotypic peptides for >99% of the predicted human proteins with matching spectral libraries, expected transitions and availability of labelled synthetic peptides (Kusebauch et al. 2016). This data can now be used to study disease-associated proteins.

In another approach, B/D-HPP has used bioinformatics to identify "priority proteins" from literature published by researchers on various organ systems with attention to cardiovascular, cerebral, hepatic, intestinal, pulmonary and renal systems (Lam et al. 2016). The data available in the SRMAtlas will be used for analysis of these proteins. This approach will be extended to study normal and disease biology.

The Chromosome HPP project (C-HPP) also involves the participation of several countries (Paik et al. 2016, 2017). There are 25 teams from across the world. The C-HPP's goal is to identify, quantify and obtain subcellular location and distributions of the predicted 20,300 proteins in a chromosome-by-chromosome manner. It is also now focussing on alternative splice isoforms and PTMs (phosphorylation, glycosylation and acetylation), small open reading frame translational products (smORFs) and long non-coding (lnc) RNAs (Paik et al. 2017). The information generated will provide data to understand basic biology and clinical conditions.

The Human Protein Atlas (HPA) programme was started in 2003 with an aim to generate antibodies to all known proteins and to utilize them to explore the function of the proteins, their interactions and cell and tissue localization. In its Version 18 released on 1 December 2017, the atlas has 26,009 antibodies targeting 17,000 unique proteins. In parallel, the atlas also provides information about the mRNA status of these proteins in major human tissues and their subcellular locales. The atlas provides an in-depth view of the proteins and their location in different tissues, in cancer and in relation to chromosomal location (http://www.proteinatlas.org). The protein data in HPA has now been accessed to generate a database of tissue- and cancer-specific biologic networks, TCSBN (Lee et al. 2018).

The knowledge resource pillar of the HPP brings together the HUPO Proteomics Standards Initiative (Deutsch et al. 2017) and ProteomeXchange repositories where the HPP investigators deposit their mass spectrometry data (Jarnuczak and Vizcaíno

2017). There are specific guidelines of how mass spectrometry data has to be deposited to the repositories (Deutsch et al. 2016). From the ProteomeXchange, the data flows to Peptide Atlas and GPMdb where the raw data and metadata is reanalysed and sent to neXtProt where the data is curated (Omenn 2017). neXtProt integrates the UniProtKB and genomics (dbSNP, COSMIC), transcriptomics (BGee), antibody-based (Human Protein Atlas (HPA), MS-based (PeptideAtlas), 3D structural information (Protein Data Bank, PDB) and posttranslational modifications from manually curated literature (Gaudet et al. 2017). Based on this, the entries are categorized into different levels of protein existence, the PE levels (Segura et al. 2017).

As per the HPP Metrics of 2017 (Omenn et al. 2017), the total predicted proteins coded for by the genome are 19,587. Of these, 17,008 have been identified with high confidence at PE1 level. Proteins labelled as "missing proteins" are 2579 in number and these have been identified at different levels of confidence at present and efforts are on to confirm their identities. The remaining 572 are dubious or uncertain proteins. The "missing proteins" arise from the observations that many proteins are in small amount and not detectable by MS. Some proteins have very low level of transcripts. The other reasons are that there are proteins lacking lysines and arginines and therefore not readily available for MS. Some proteins are not readily soluble, such as the membrane proteins, and some proteins are present only in specific conditions. All these situations do not permit easy detection of the proteins (Segura et al. 2017).

It is expected that each of the HPP projects will generate information to contribute to completing the human proteome information in the near future.

14.3 Proteomic Tools for Biological Investigations

14.3.1 Separation Methods

14.3.1.1 One-Dimensional and Two-Dimensional Polyacrylamide Gel Electrophoresis

The major proteomic tools for understanding a biological process comprise some very *familiar biochemical techniques which are tailored for high-throughput analysis*. Figure 14.2 shows how a sample can be processed for identification of the proteins therein. These techniques are described in this section. The main aim of proteomics is to first determine the number of proteins in the system under study. For this, total proteins in a sample are separated by one- and/or two-dimensional SDS polyacrylamide gel electrophoresis (1D/2D-SDS-PAGE) (Magdeldin et al. 2014). In 1D-SDS-PAGE, proteins are separated on the basis of their mass (Fig. 14.3). In 2D-SDS-PAGE, proteins are first separated on the basis of their isoelectric point (pI) using isoelectric focussing (IEF) gel strips of different pH ranges. The strip containing proteins separated on the basis of their pI is then placed horizontally on an SDS-containing polyacrylamide gel and subjected to electrophoresis. The proteins now separate according to their molecular weight (Fig. 14.4). The

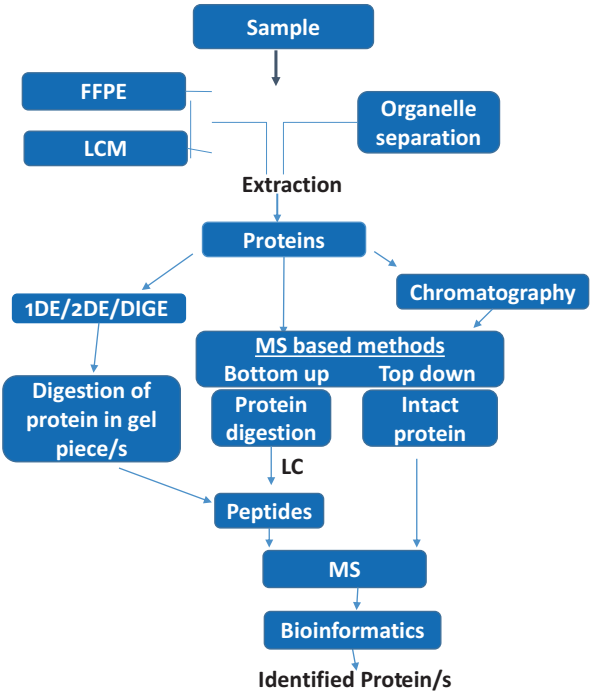

Fig. 14.2 Flow diagram of the various separation tools and prefractionation methods used for the identification of protein(s) in a sample. *FFPE* formalin-fixed paraffin-embedded sample, *LCM* laser capture microdissection, *1DE* two-dimensional gel electrophoresis, *2DE* two-dimensional gel electrophoresis, *DIGE* differential gel electrophoresis, *LC* liquid chromatography, *MS* mass spectrometry

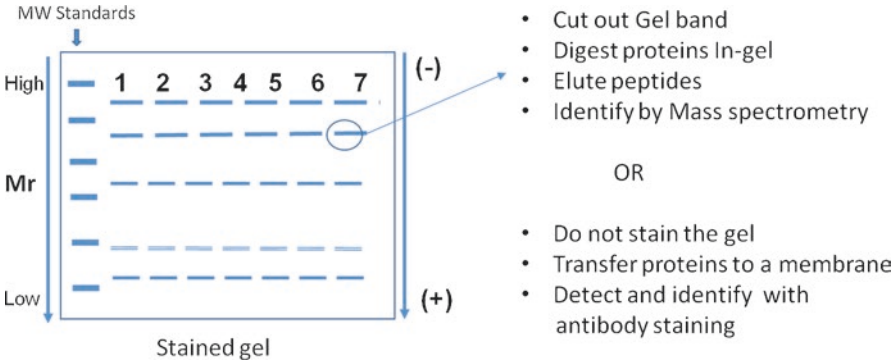

Fig. 14.3 One-dimensional sodium dodecyl sulphate gel electrophoresis (1DE). An acrylamide gel containing sodium dodecyl sulphate (SDS) is made with several wells (e.g. 1–7). Different protein samples are loaded in each of the wells and resolved by applying a voltage across the gel. The proteins separate on the basis of their molecular weight. Proteins of known molecular weight (MW) are loaded in a parallel lane. After the electrophoresis, the proteins in the gel are detected by staining. The relative molecular weight (Mr) of the proteins is obtained by comparing with the mobility of the standard proteins. The protein(s) in the gel bands can be identified by mass spectrometry. The proteins in such a gel can also be transferred before staining to a membrane to detect presence of specific proteins using antibodies

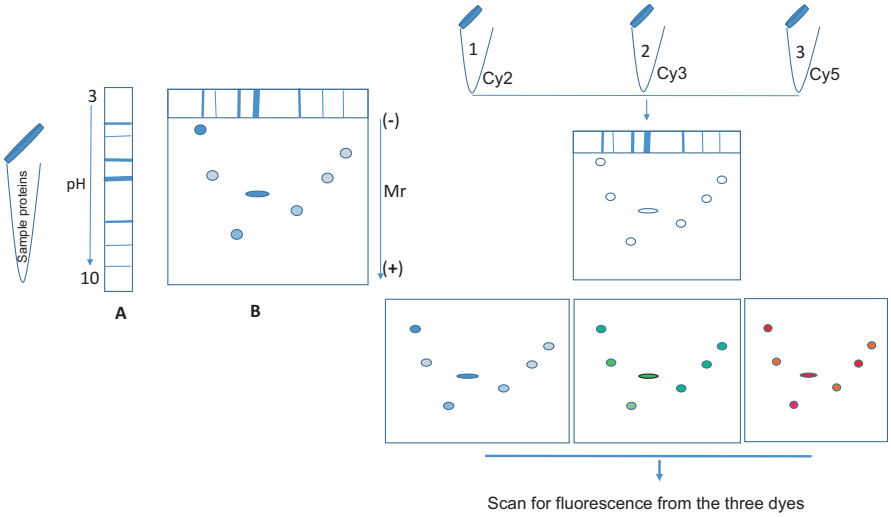

Fig. 14.4 2DE-differential gel electrophoresis (DIGE). (**a**) For two-dimensional gel electrophoresis (2DE), the sample proteins are first separated on the basis of their pI. (**b**) The strip containing the separated proteins is laid horizontally on a polyacrylamide gel containing sodium dodecyl sulphate (SDS) and the proteins separated according to their molecular weight (Mr). The gel is then stained to visualize the proteins. For DIGE, the proteins in the three samples 1, 2 and 3 are each labelled with one of the fluorescent dyes Cy2, Cy3 and Cy5. The labelled proteins are mixed and resolved by 2DE. The gels are then scanned at different wavelengths to determine the intensity of staining of each of the proteins. The relative concentration of each of the proteins in the three samples is obtained by comparing the fluorescence intensity using specific software

proteins resolved by 1D- or 2D-SDS-PAGE are detected by staining and then visualized for quantification. Staining technologies include staining with Coomassie Brilliant Blue, silver staining, SYPRO Orange and others (Chevalier 2010). Prior to staining, the proteins can also be transferred to membranes and detected by immunoblotting as described below in Sect. 14.3.4.1.

To enhance their detection and quantitation, proteins can be labelled with fluorescent reagents before electrophoretic separation. The reagents used are three fluorescent dyes, Cy3, Cy5 and Cy2, which are size-matched, charge-matched and spectrally resolvable at 488 nm (Cy2), 532 nm (Cy3) and 633 nm (Cy5) (Timms and Cramer 2008). The dyes carry an N-hydroxylsuccinimidyl ester reactive group that covalently reacts with the e-amino groups of lysine residues in the proteins. The labelling and subsequent separation technique is called *difference gel electrophoresis (DIGE)*. It provides a method to separate proteins from three different samples on the same gel. For example, proteins from a normal sample can be labelled with Cy3, diseased sample with Cy5 and a 1:1 mixture of normal and diseased sample with Cy2 as an internal control. The labelled samples are then resolved on a single gel and scanned at different wavelengths to determine the amount of the resolved proteins in each sample using appropriate software (Fig. 14.4). This method of labelling overcomes experimental errors, ensures increased sensitivity and reproducibility and reduces inter-gel variability (Wong et al. 2009).

The proteins which are separated by electrophoresis and stained are then punched out for further processing. The proteins in the punched-out gel are digested with an enzyme which is generally trypsin. The peptides generated are eluted and characterized by mass spectrometry for identification and quantification.

14.3.1.2 Liquid Chromatography

A protein digest generated from a source tissue or biofluid can also be separated by liquid chromatography, and the fractions emerging can be analysed by mass spectrometry (Fig. 14.5). In this method, the peptides are resolved on columns based on their size, charge, polarity or hydrophobicity. The methods are referred to as size exclusion chromatography, ion exchange chromatography, hydrophilic interaction liquid chromatography and reverse phase chromatography, respectively. The resolution of the peptides is determined by the characteristics of the column and the mobile phase (Manadas et al. 2010).

14.3.1.3 Formalin-Fixed Paraffin-Embedded (FFPE) Tissue

If tissue is available in a paraffin block made for histopathology, proteins can be recovered from the block and processed for digestion followed by liquid chromatography and mass spectrometry for identification (Magdeldin and Yamamoto 2012).

14.3.1.4 Laser Capture Microdissection

In this method, specific cells in a tissue section can be selectively removed by etching out the required area of the tissue fixed on a slide using a laser capture microdissection microscope. The selected cells are then transferred to a microcentrifuge tube

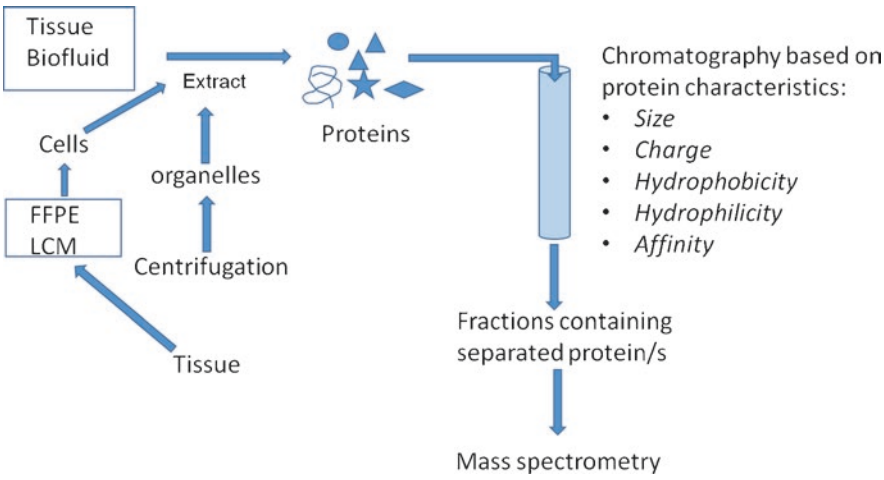

Fig. 14.5 Prefractionation to reduce the complexity of samples. Proteins are extracted from tissues, biofluids, organelles separated by centrifugation and cells derived from formalin-fixed paraffin-embedded (FFPE) blocks or from laser capture micro dissected (LCM) tissue sections. These proteins are separated by chromatography based on their characteristics. The separated proteins are then identified and quantitated by mass spectrometry

for further processing (Espina et al. 2006). The identity and quantity of the cellular proteins is obtained by digestion and mass spectrometry as given above.

14.3.2 Prefractionation

The complexity of the proteins (number and amount) and the technological hurdles to be addressed to obtain good separation has now necessitated the need to pre-fractionate a mixture of proteins (Hanash et al. 2012; Larance and Lamond 2015; El Rassi and Puangpila 2017) (Fig. 14.5). These pre-fractionation methods could include separation of subcellular organelles by centrifugation and then processing for protein identification. Other methods include solubilization of a tissue with buffers and fractionation of the mixture of proteins on columns based on charge, polarity, hydrophobicity, size, affinity chromatography, etc., and then analysing them by mass spectrometry. Proteins with specific posttranslational modifications such as phosphorylation and glycosylation can be separated by affinity chromatography and then studied by mass spectrometry. Specific proteins, single or associated with others, could be separated by antibody affinity chromatography. Membrane proteins can be tagged with specific reagents and the membrane lysates then separated by affinity chromatography specific for the tags. Similarly, proteins in the secretome or condition medium can be specifically analysed by gel-based or gel-free techniques. The end point in all these techniques is mass spectrometry for identification of the protein composition in the sample.

14.3.3 Mass Spectrometry

This is a technique wherein the peptides in the protein digests are separated based on their mass and charge. There are a variety of mass spectrometers (Aebersold and Mann 2003) (Fig. 14.6). All of these have an ion source, an analyser and a detector. For proteomics, the ion source used is electron spray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI). In ESI mode, the peptide digest is inserted as a spray of droplets into the analyser where the peptides are ionized. In the MALDI equipment, the peptide digest is co-crystallized with a chemical on a matrix which promotes ionization when bombarded with a pulse laser which has a wavelength of light which is absorbed by the chemical. The ionized peptides are then separated on their mass-to-charge ratio (m/z) through different formats of analysers such as the time-of-flight (TOF), or quadrupoles (QD), or hybrid quadrupole-time-of-flight mode (QD-TOF), ion trap (IT) and Fourier transform ion cyclotron resonance (FTICR). Different analysers are sometimes coupled together for better performance. The number of ions with specific m/z is then recorded by a detector. The peak profiles generated are compared with the information available in various peptide mass spectrometry libraries using search engines such as MASCOT, Protein Prospector and ExPASy, and the identity of the proteins in the mixture is deciphered (Baldwin 2004). Each equipment has specific sensitivity and resolution capabilities,

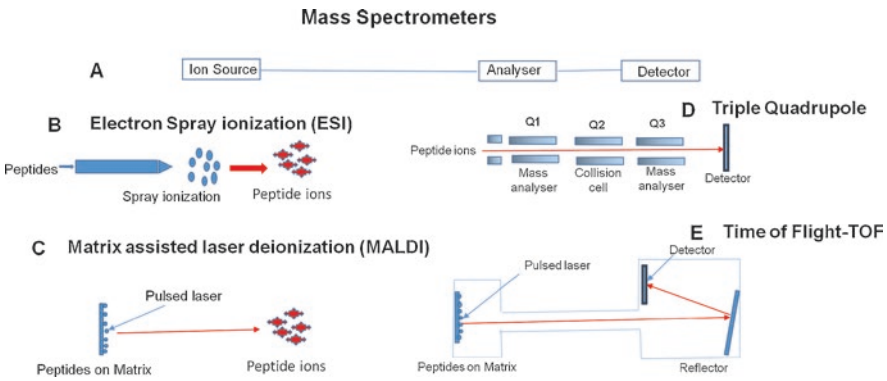

Fig. 14.6 Mass spectrometers. The figure shows the parts of a mass spectrometer A and the ionization modes, B and C, which generate the peptide ions and two formats of mass analysers with detectors, i.e. the triple quadrupole and the time of flight, D and E, respectively. Q1, Q2 and Q3 are the quadrupoles

and appropriate machines have to be used as per the requirements of the biological query.

Mass spectrometry as described above is referred to as a *bottom-up* approach. In this, a protein or mixture of proteins is digested into peptides, separated through liquid chromatography and then subjected to identification and quantification by mass spectrometry. In another approach, *top-down*, a protein or mixture of proteins is separated by liquid chromatography and then subjected to fragmentation into peptide ions which are then separated in the mass analyser and the spectral profile recorded by a detector (Fig. 14.2).

The ion capture in the mass analyser can be done in the data-dependent acquisition (DDA) or data-independent acquisition (DIA) mode (Hu 2016). In DDA, the most *abundant* precursor peptide ions are identified in the survey scan for further fragmentation and identification. This mode is generally used for discovery proteomics or shotgun proteomics wherein the digested proteins in a mixture are separated through liquid chromatography and then passed through a mass spectrometer for detection and identification. This is referred to as LC-MS/MS in which there are analysers in tandem. In the mass spectrometer, first the precursor peptides are ionized; then, in the MSI scan, the abundance and mass-to-charge ratio of all the ions eluting at a given time are measured, and thereafter some or all detected ions are fragmented, and in the MS2 scan, the abundances and m/z's of the fragments are measured and recorded. For the DIA mode, *all* the generated precursor peptide ions are scanned sequentially over selected mass ranges so that all the ions are detected and can be further fragmented for precise identification. The data emerging is more complex to analyse.

For quantitation, validation and reproducibility of *specific* proteins, selected reaction monitoring (SRM) also known as multiple reaction monitoring (MRM) method is used (Lange et al. 2008). It is also known as targeted data acquisition. It is classically done in a triple quadrupole equipment. The precursor peptide ion

specific for a protein of choice is selected in the first quadrupole. It is fragmented in the second quadrupole and a single fragment ion of choice evaluated in the third quadrupole. If multiple fragment ions of the specific precursor are evaluated, it is referred to as parallel reaction monitoring (PRM). Targeted data acquisition is a very sensitive method for *absolute quantitation*.

Relative quantitation of proteins/peptides can be assessed by mass spectrometry using specific protein labelling techniques such as SILAC, ICAT and iTRAQ and enzymatic labelling of proteins/peptides. SILAC (stable isotope labelling of amino acids in culture) is used for metabolically *labelling proteins* in cells in culture with heavy isotopes of C, N, H or O such as C^{13}, N^{15}, H^{2} and 0^{18}. Cells to be compared are grown in culture medium containing either the heavy isotope-labelled arginine or lysine or the regular medium. The labelled proteins in the cells are then processed for separation, digestion and mass spectrometry. A small shift in the mass spectrometry signal due to the presence of the heavy isotope in a peptide can be identified and the amount of the peptide ion quantitated (Mann 2006; Ong et al. 2002; Ong and Mann 2006).

A method of *labelling peptides* is the isobaric tagging reagent for absolute quantification (iTRAQ). iTRAQ has four reagents, each of which has a reporter group (with variable mass of 114–117 Daltons), a balance group (with variable mass of 31–28) and an amino reactive group which reacts with lysine side chains and amino terminals. The variable masses are obtained by presence of different numbers of isotopes of C, O and N in the reporter and balance groups. The combined mass of the reporter group and the balance group of each of the four reagents is 145 Daltons. The proteins in the samples to be compared are first digested with trypsin. The peptides generated in each of the samples are labelled with one of the four reagents Fig. 14.7. The tag reacts with the N terminus and the e-amino group of lysines in the peptides to form an amide bond which is fragmented during collision-induced dissociation in the second quadrupole, to release the reporter ion. The labelled peptides in the samples to be compared are pooled and fractionated by strong cation exchange (SCX) chromatography, and each desalted fraction is subjected to tandem mass spectrometry. Since the tag is isobaric, the peptides with the tag elute at the same retention time in liquid chromatography and have the same m/z value in the MS1 scan. When the tagged precursor ion is fragmented at the amide bond, it generates two types of product ions. They are (1) the reporter ion and (2) the peptide ion used for identification. The reporter groups of the iTRAQ reagent generate reporter ions for each sample with mass/charge (*m/z*) of 114, 115, 116 and 117 which are detected during the MS2 scan. The ratio of the intensity of the reporter ion to that of the peptide-specific ion is used for quantitation. The intensities of the reporter ions allow the differentiation of the different samples in MS and furnish the necessary quantitative information (Rauniyar and Yates 2014).

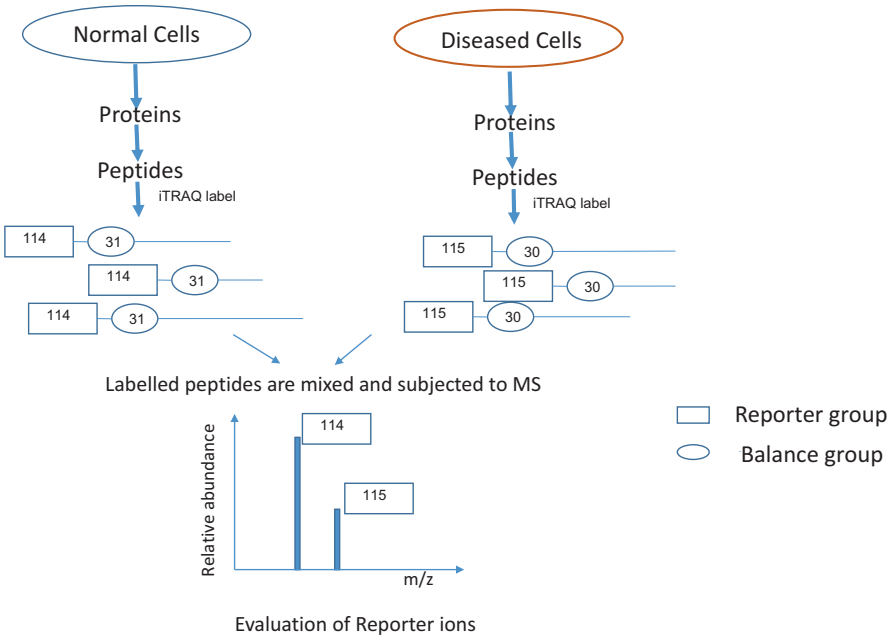

Fig. 14.7 The use of iTRAQ method for quantitation of proteins. Peptides generated from normal and diseased cells are labelled with the iTRAQ reagent with different reporter groups. The labelled peptides are mixed and subjected to mass spectrometry. The intensity of the reporter groups indicates the amount of each protein in the two samples

14.3.4 Verification of Identity

The identity of the proteins determined by mass spectrometry is confirmed by other techniques, such as Western blotting, tissue arrays and microarrays which require a specific antibody to detect the protein identified.

14.3.4.1 Western Blotting

The identity of a protein obtained by mass spectrometry can be confirmed by Western blotting (Kurien and Scofield 2015) (Fig. 14.8). In this method, proteins separated on a ID/2D-SDS-PAGE are electrophoretically transferred to a membrane. The membrane is blocked with a buffer containing a reagent to reduce non-specific binding. The blocked membrane is again washed with a buffer without the blocking reagent. The protein on the membrane is then detected by reacting with an antibody specific to it. The bound antibody is detected with a secondary antibody tagged to an enzyme such as horseradish peroxidase or alkaline phosphatase. The position of the identified protein is detected using reagents containing a substrate for the enzyme which produces a colour or a chemiluminescent signal. The signal obtained is then recorded. If several different samples containing the protein are to be evaluated, the samples are loaded in separate lanes of a gel. The proteins in the

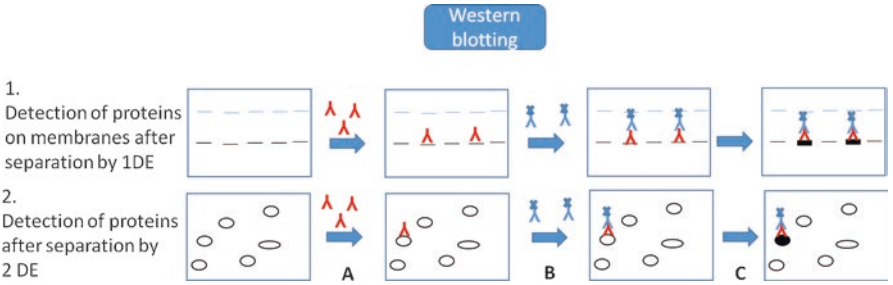

Fig. 14.8 Steps for detecting proteins which have been transferred onto membranes after 1DE or 2DE (one-dimensional and two-dimensional gel electrophoresis). 1. Multi-Western blot, the blot shows several lanes in which protein mixtures from different samples have been separated by 1DE and transferred to a membrane. 2. A blot in which a protein mixture has been separated by 2DE and transferred to the membrane. For both the blots, in step A, the blots are probed with a primary antibody for the protein which has to be identified from among the proteins which have been separated. In step B, the antibody bound to the specific protein is detected with a secondary antibody which is tagged to an enzyme. In step C, substrate for the enzyme is added. After the reaction, a coloured reagent is deposited at the position of the detected protein (shown here as a darkened band/spot). The intensity of the coloured deposit determines semiquantitatively the concentration of the detected protein

samples are separated by electrophoresis and transferred to a membrane which is then probed with the antibody as above. This method is referred to as multi-Western blotting.

14.3.4.2 Multiple Protein Identification and Quantification by Array Techniques

Microarrays are being used to evaluate several different protein-related queries (Chandra et al. 2011; Atak et al. 2016; Liotta et al. 2003). There are varied formats of arrays. In each of these, either antibodies or protein/s are spotted on a suitable support such as a membrane or slide in an array format. A few of these are given below.

Antibody Arrays

Antibodies to different proteins are arrayed on supports (Fig. 14.9). They are then interacted with the protein/peptide mixture in which the identity and quantity of different proteins have to be determined. The proteins which interact with the spotted antibodies are then detected with another antibody to the protein, tagged with a detection reagent. The colour/fluorescent signals obtained from the tag are quantitated by appropriate scanning equipment. Figure 14.9 shows the step-by-step procedure. Antibody arrays can be used to assess protein–protein interactions, posttranslational modifications on proteins, autoantibody responses, etc.

Reverse-Phase Protein Arrays

In this method, *cell lysates* from different cells or laser capture microdissected cells or body fluids are spotted on to a microarray (Creighton and Huang 2015). After

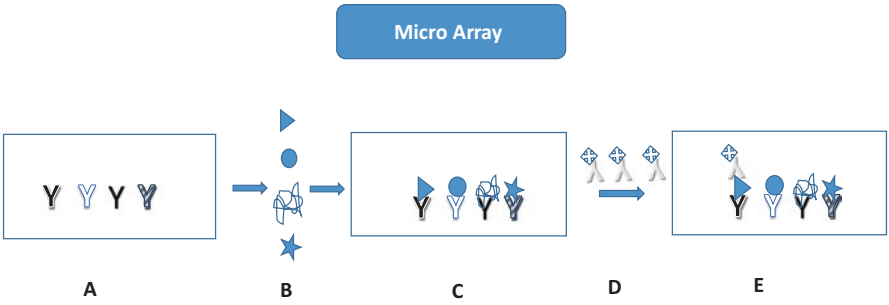

Fig. 14.9 An antibody array for detecting and quantitating proteins. Antibodies to different proteins are first arrayed on a support. (**a**) shows a single row of different antibodies. A mixture of proteins (**b**) is layered over the antibody array. The proteins bind the respective antibody which detects them (**c**). The protein of interest in the mixture is detected with a secondary antibody to it tagged to a fluorescent molecule (**d**, **e**). The intensity of the fluorescence is measured with a detector, and the output determines the concentration of the bound protein. In *reversed-phase arrays*, different cell lysates are spotted on the array instead of antibodies, and the array is overlaid with a primary antibody to a specific protein which is to be identified and quantified in the lysates. The primary antibody is then detected by reacting with a tagged secondary antibody

optimal blocking of unoccupied sites, the spotted arrays are incubated with an antibody to a protein whose presence and concentration is being evaluated in the cells/biofluid. The bound antibody or a secondary labelled antibody is detected by chemiluminescent, fluorescent or colorimetric assays (Fig. 14.9).

Tissue Arrays

The presence of a protein in a *tissue section* can be determined and its amount quantitated using tissue arrays (Kononen et al. 1998; Torhorst et al. 2001) (Fig. 14.10). For this, tissues fixed in paraffin blocks for histopathology assessment are used. Cores containing defined areas from the fixed tissue in the donor paraffin block are removed and transferred to a specific spot in an array format on a recipient paraffin block. This process is repeated for different tissues from several donor paraffin blocks. The recipient block is then cut to yield ultrathin tissue sections of the arrayed cores which are placed on a slide and processed for immunohistochemical evaluation with an antibody. The presence and amount of protein as determined by the coloured stain is assessed by microscopy.

This technique is useful to investigate a protein in several different tissues simultaneously. It provides information about its presence, amount in semi-quantitative terms and location within the cell. A large number of different tissue sections can be evaluated for a single protein on a single slide, and this can be repeated for the detection of different proteins with specific antibodies.

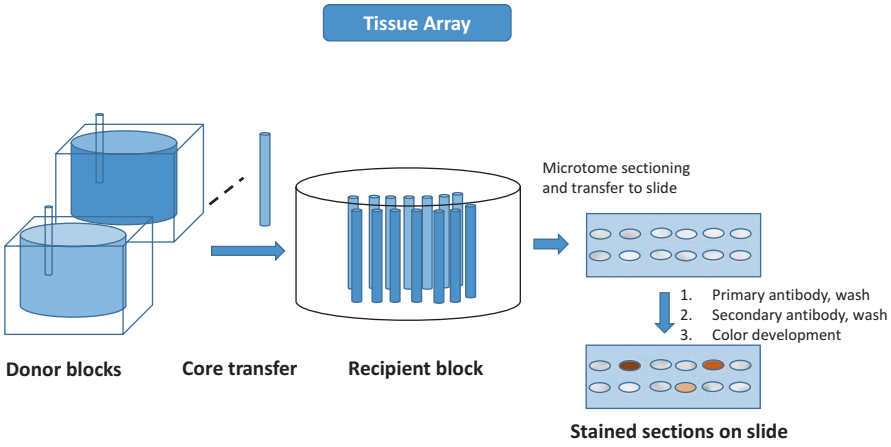

Fig. 14.10 Tissue array for detecting the presence of a protein and determining its quantity in the sample. Tissues are fixed in paraffin blocks. Cores are taken out from the donor blocks and transferred to the recipient paraffin block in an array format. Ultrathin sections of the tissues from different donor blocks are then cut from the array in the recipient block and transferred to a slide. The tissue sections are processed for staining, and the sections which have the protein are identified from the colour seen in the sections. The intensity of the stain provides a semiquantitative estimate of the protein in the tissue. Tissues from different samples can be processed at a time for detecting and quantitating a specific protein

14.3.5 Phosphoproteomic Profiles

In many of the signalling cascades such as those for growth and apoptosis, the signalling molecules interact with cell surface molecules and stimulate cytosolic proteins, many of which are kinases and phosphatases. The aberrant phosphorylation or dephosphorylation of key molecules within the cascade could lead to transformation. Assessment of the phosphorylation status is therefore an important study which is undertaken using proteomic tools.

Proteins from normal and diseased tissues are first solubilized in appropriate buffers. Phosphoproteins are in low abundance and, therefore, there is need to enrich these molecules by different techniques. Affinity chromatography methods for enrichment of phosphorylated peptides/proteins include use of immobilized metal affinity columns (IMAC) (Andersson and Porath 1986) and of TiO2 (Larsen et al. 2005). Phosphopeptides/proteins can also be enriched on affinity columns which are linked to antibodies against specific phosphorylated proteins/peptides. The enriched phosphopeptides/proteins are then analysed by mass spectrometry (Harshaa and Pandey 2010). This exercise can also be done without separation of the phosphoproteins/peptides by chromatography as above. In this case, the complexity of the spectral patterns will be a challenge to decipher.

In another approach, the protein mixture is layered over an antibody array which detects phosphoproteins (Atak et al. 2016; Liotta et al. 2003). The signals obtained therefrom would provide information about the proteins which have undergone

phosphorylation or dephosphorylation. This approach however requires prior knowledge of proteins which are likely to undergo phosphorylation/dephosphorylation. The antibody arrays or the direct assessment of phosphoproteins by mass spectrometry can be used to follow alterations in signalling cascades in response to drug action. EGFR is known to be overexpressed in several cancers. This leads to activation of the growth factor related signalling cascades. Drugs to inhibit action of EGFR are known and are used for therapy. It is possible to analyse the alteration in protein phosphorylation with and without the drug using the proteomic tools described. If a tumour tissue does not respond to the drug, evaluation of the phosphoprotein profile will provide a clue to which proteins are not undergoing the expected phosphorylation/dephosphorylation process. Such profiles are now being used to provide reports for cancer management.

14.3.6 Bioinformatics

The data emerging from proteomics investigations is large and is referred to as big data. This is available in several databases which are readily accessible. The HPP data is in the information given above. Some of the other databases are UniProt, Swiss-Prot protein knowledge base, PROSITE database of protein families and domains, PDB-protein structure database, DIP-database of interacting proteins (http://dip.mbi.ucla.edu/dip/), Ingenuity pathways, KEGG pathway, PANTHER, etc. These databases provide links from and between basic sequence information, mutations, structure, interactomes and pathways. This list is only a few of the many databases useful for protein-related queries.

14.4 Proteomics for Signalling Networks in Cancer

Cancer is a disorder arising from alterations within the genome leading to uncontrolled proliferation, evasion of growth suppression reduced apoptosis, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming of energy metabolism and evasion of immune destruction (Hanahan and Weinberg 2011). Each of these characteristics is due to aberrant signalling mechanisms.

Different types of changes in the molecules in the signal cascade have been reported. They include increased expression of cell surface receptors, loss of extracellular domain of the receptor, mutations in the molecule, alterations in the functions of other molecules in the signal cascade due to mutations and altered expression. The changes in the molecules lead to altered signal transduction arising from changes in phosphorylation/dephosphorylation.

Although immense amount of information on components of the signalling cascades is emerging and several key components have been identified, it is apparent that cancer is a complex disease. It is therefore necessary to identify as many proteins which contribute to the transformation so that the complexities arising due to

heterogeneity of cell types in a tissue and clinical presentation of each individual with cancer in a specific organ can be addressed towards personalized medicine for better patient management. With the advent of proteomic technologies, it has become possible to undertake this immense task.

The approaches that have been taken are initially in the *discovery mode* wherein normal and tumour tissues/biofluids have been processed for SDS-PAGE/liquid chromatography and mass spectrometry for identifying the number and quantity of proteins in the mixture. This is followed by analysing the data using high-end statistics and bioinformatics to delineate the number of altered proteins and their involvement in different signalling cascades. Through such investigations, shortlists of proteins have been made which are potential key candidates for a signature which can distinguish the transformed sample from the normal. The challenges in this approach are to generate the optimal set of proteins representative of the cancer. The data obtained is evaluated in terms of the signalling pathways that the proteins belong to and further permit a meaningful assessment of key proteins which can be used for cancer management.

Proteomic tools are now being used to address specifically, risk assessment, diagnosis, prognosis, prediction, invasion and metastasis and response to therapy. For each of these, the choice of the appropriate clinical sample and overall study design and the technologies that are to be used is important.

Plasma proteomics has been receiving attention for biomarker discovery as blood can be obtained with less invasive techniques. However, the protein composition of plasma is very complex, and it contains proteins in varying amounts of ten orders of magnitude from milligrams to femtograms per ml (Geyer et al. 2017). Due to this complexity, it is a challenge to identify disease-related biomarkers as they may be lost among the abundant proteins. Fractionation of subcellular particles is being used to overcome these hurdles.

Extracellular vesicles (EV) in plasma are reported to host proteins representative of disease. These protein markers could be used for *early diagnosis of disease*. Vykoukal et al. (2017) have investigated the protein composition of extracellular vesicles enriched from plasma of individuals with early adenocarcinoma of the lung and normal controls. They have used a single-step flotation density gradient ultracentrifugation method to obtain pure populations of EV free from protein aggregates and other soluble proteins. The EVs so obtained were characterized by transmission electron microscopy and particle size measurements. The protein content in the EVs was determined and then analysed by 1DE-SDS-PAGE and Western blotting using antibodies to an endosomal sorting marker TSG1. The proteins in the enriched vesicle preparation were then resolved by 1DE-SDS-PAGE. The gel was cut into pieces and the proteins within the gel subjected to trypsin digestion. The peptides were extracted and separated by nano HPLC, and the resolved peptides were analysed by mass spectrometry and the identity of the proteins obtained by searching on the Uniprot human database. Bioinformatic analysis revealed that the EV from plasma of patients has differentially expressed proteins which are involved in pathways that affect altered metabolism, protein fate and trafficking, cytoskeletal

remodelling, transcriptional regulation and tumour stemness which could be considered for further clinical evaluation.

Detection of early-stage disease improves prognosis and the management of cancers. Yoneyama et al. (2016) have used a combination of antibody-based and LC-MS/MS-based proteomic techniques to identify markers in plasma for the detection of early stage of pancreatic cancer. By using combination of proteomic tools, it is possible to enrich for proteins which could be considered for early detection. In this study, the authors first used 260 genes which had been shortlisted by Nakamura et al. (2004) as those which were upregulated in invasive ductal adenocarcinoma of pancreatic (IDCAP) cells compared to normal pancreatic ductal epithelial cells using genome-wide cDNA microarray technique and microdissected tissue.

Plasma samples were collected from patients from seven different institutions participating in the project. The patients were classified as per the approved TNM classification for the disease according to the UICC criteria. Healthy controls were individuals with benign disease or those who had gum disorders or implants.

The first proteomic tool for identifying disease markers was reverse-phase protein array (RPPA), as antibodies were available for 130 proteins of the 260 shortlisted genes identified for further evaluation. RPPA is a highly sensitive method, and it permits evaluation of a large number of plasma samples. Plasma samples were diluted serially (8-, 16-, 32- and 64-fold) and spotted on a ProteoChip glass slides (Proteogen, Seoul, Korea) in quadruplicate in array format using a protein microarrayer in which 384 spotted samples could be assessed simultaneously. The slides were blocked with PBS containing 0.5% casein, and each slide was then incubated with one of the 130 antibodies to detect the presence and quantity of the respective protein. The slides were washed and the bound antibody detected with a secondary antibody tagged with biotin and subsequently treated with streptavidin conjugated to horseradish peroxidase for a fluorometric detection assay.

To improve the specificity of detection of a protein in the plasma samples and to quantitate it, SRM/MRM method of mass spectrometry was used. The authors developed an in silico method to identify a target peptide for each protein which could be used to follow by mass spectrometry. They also developed an automatic sample processing system, micro LC and autoanalysis system to permit a high-throughput evaluation of a large number of plasma samples. The proteins in the plasma samples were digested with trypsin, and the selected peptide quantitated by SRM/MRM analysis.

For the RPPA analysis, 106 plasma samples from healthy controls and 164 samples from stage 1 and II IDCAP patients were used. Twenty-three proteins were chosen from the RPPA analysis as candidates for the SRM/MRM assay. Of these, complement C2 (C2b) and insulin-like growth factor-binding protein 2 (IGFBP2) were optimally detected and quantitated in the plasma samples. The authors also chose related proteins C2a, IGFBP3, CRP and adiponectin which have a role in cancer risk and inflammation for the SRM/MRM assay.

The five proteins (complements C2b and C2a, IGFBP2, IGFBP3 and CRP) were quantified in an early-stage set, which was composed of 38 stage I or II IDACP

patients and 65 healthy controls, in order to validate these proteins as early-stage IDACP biomarkers. The quantitative values of IGFBP2 and IGFBP3 of patients were found to be significantly greater and lower, respectively, than those of the controls. There were no significant differences for the other biomarker candidates between patients and controls, indicating that these proteins are not useful biomarkers for detection of early-stage IDACP.

The levels of IGFBP2 and IGFBP3 were assessed in patients who were negative for CA19-9 which has been used as a marker for pancreatic cancer (Ballehaninna and Chamberlain 2011). IGFBP2 and IGFBP3 were shown to have compensatory ability for CA19-9 in the diagnosis of IDACP. Measurement of all these markers can thus improve diagnostic performance. With the use of antibody- and mass spectrometry-based high-throughput proteomic tools, the authors have defined markers which can be used to screen plasma from individuals with early-stage pancreatic cancer.

Proteomics is being used for identifying key molecules within signalling cascades which could help in the assessment of *progression of cancer*. Mori et al. (2017) have used the iTRAQ methodology to identify markers to determine lymph node (LN) metastasis in patients with colorectal cancer (CRC). In CRC, due to LN metastasis, the 5-year survival rate of the patients is lowered (Siegel 2012). Good biomarkers are needed in preoperative samples for prognosis of metastasis so that optimal treatment can be given to the patients who are likely to have LN metastasis and those who will not. The authors have compared four samples consisting of colorectal cancer tissue from patients with or without LN metastasis, the adjacent normal colon mucosa and a sample from normal colon mucosa. For the initial discovery proteomic investigations, five samples of each category were used. Proteins in the first set of four samples were extracted and processed for digestion with trypsin. The peptides generated from each sample were labelled with one of the four iTRAQ reagents with tags 114, 115, 116 and 117. These were then mixed and passed through a cation exchange cartridge system prior to LC-MS/MS. The data obtained from the mass spectrometer was analysed using the ProteinPilot software. The experiments were repeated for five such sets of four samples. Four thousand differentially expressed proteins between the four samples were obtained. From this, a shortlist of 55 proteins was made using specific criteria. Using the Panther software, these proteins were categorized as per their molecular function, cellular component and biological processes. Out of this, four proteins were further evaluated. These belong to the development process which includes metastatic processes such as the epithelial-mesenchymal transition. The four proteins are ezarin, tropomyosin alpha-3 chain, cytokeratin 18 and interleukin enhancer-binding factor 3.

In another experiment, 45 fresh-frozen samples, consisting of 14 CRC with LN metastasis and 14 CRC without LN metastasis, 17 samples of normal colon mucosa were evaluated for mRNA expression of the four shortlisted proteins. The level of ezarin mRNA was higher in CRC samples with LN metastasis compared to those without LN metastasis and the normal colon mucosa. The levels of the mRNA for the other three proteins did not differentiate normal colon mucosa, CRC with and without LN metastasis.

The data was validated in two cohorts of patients. In one cohort, the tissues in the FFPE blocks of 195 patients with CRC were analysed for ezarin protein by immunohistochemistry. In the second cohort, fresh-frozen surgical samples from 170 patients with CRC were evaluated for mRNA expression of ezarin by real-time PCR. The data obtained for protein and mRNA expression was statistically evaluated along with clinical criteria of the patients. It was found that protein and mRNA levels are both independent predictors of LN metastasis. Since mRNA levels can be more quantitatively evaluated, it could be used for assessing pre-surgical samples of the patients for ezarin mRNA and decide the course of treatment.

One of the well-studied cell surface receptors is the epidermal growth factor receptor *EGFR* which is located on the plasma membrane of cells. It belongs to the ErbB family of receptor tyrosine kinases (Eccles 2011) and regulates epithelial cell functions. Ligand binding to EGFR stimulates an extremely complex signal cascade from the plasma membrane to the nucleus via several cytosolic signalling molecules, leading to the activation of genes which regulate cell proliferation, survival and differentiation. On binding of EGF to the receptor, the receptor dimerizes, which is followed by trans autophosphorylation of tyrosine residues in the cytoplasmic tail kinase domain. This activation is then transduced to different signalling pathways including the Ras/MAPK pathway, the PI3K/AKT pathway and the phospholipase C (PLC)/protein kinase C (PKC) signalling cascade. The structure of the receptor and signalling pathways stimulated from it are comprehensively described by Sigismund et al. (2018) and Wee and Wang (2017).

EGFR consists of an extracellular domain which is involved in ligand binding and homo- or hetero-dimer formation with analogous domains of family members. The transmembrane domain (TM) of the receptor anchors it to the membrane. The intracellular domain has a flexible juxta-membrane segment, the tyrosine kinase domain and the C-terminal tail. The kinase domain contains lysines which are sites for ubiquitination. The C-terminal tail contains several tyrosines which when phosphorylated on receptor activation provide sites for binding of intracellular molecules which then regulate the signalling cascades.

EGFR is the target of multiple cancer therapies as it is frequently mutated and/or overexpressed in different types of human cancers. Mutations in EGFR are reported to occur in the extracellular region, the kinase domain and the C-terminal tail (Pines et al. 2010). For example, in most glioblastomas, there are aberrations in the ectodomain, whereas in non-small cell lung cancers (NSCLCs), mutations are almost exclusively located in the kinase domain (Lee et al. 2006; Pines et al. 2010). EGFR is also overexpressed in NSCLC (Veale et al. 1987). Most mutations and truncations in EGFR promote its constitutive activation by stabilizing ligand-independent dimerization with ErbB family receptors (Brewer et al. 2013). Some mutations allow the receptor to escape downregulation by endocytosis (Gan 2013).

In the kinase domain, the most commonly seen EGFR point mutation is L858R (Shigematsu et al. 2005). Other activating mutations in the kinase domain are the various EGFR exon 19 in-frame deletions which are commonly observed in NSCLC (Mitsudomi and Yatabe 2010). Another kinase domain mutant, T790 M, confers

resistance to pharmacological EGFR-tyrosine kinase inhibitors (Zhang et al. 2010; Yun et al. 2008).

The overexpression of EGFR and genetic alterations in different parts of its structure in several cancers has led to the development of two major classes of EGFR-targeted therapies. One class of therapy includes humanized monoclonal antibodies against the EGFR extracellular domain. These antibodies block ligand binding or mediate its downregulation (Martinelli et al. 2009). The other class are molecules which are ATP mimetics that bind to the receptor's kinase pocket and inhibit tyrosine kinase activity of EGFR. These molecules are referred to as tyrosine kinase inhibitors (TKI). They prevent ATP binding and in turn signal transduction (Ciardiello and Tortora 2008). EGFR monoclonal antibodies include cetuximab (Erbitux) and panitumumab (Vectibix), and approved TKIs include erlotinib (Tarceva), gefitinib (Iressa) and lapatinib (Tykerb).

In a recent publication, Jacobsen et al. (2017) have used proteomic technologies to understand the *resistance to drugs arising in NSCLC*. In this cancer, 17% of the tumours harbour the mutations in epidermal growth factor receptor (EGFR) (Rosell et al. 2009), which are deletions in exon 19 (delE746-750) or substitution of arginine for leucine (L858R) in exon 21 in the tyrosine kinase domain which confers sensitivity to the EGFR-tyrosine kinase inhibitors (TKIs) gefitinib (Maemondo et al. 2010), erlotinib (Rosell et al. 2012) or afatinib (Sequist et al. 2013). However, in 70% of the cases, the patients acquire resistance to the TKIs after treatment (Rosell et al. 2012). Several different mechanisms have been described which account for the resistance, of which the most frequent mechanism of resistance to EGFR-TKI treatment is the secondary mutation in exon 20 of EGFR, T790 M (Kobayashi et al. 2005). However, which mode of resistance will be seen in each patient is unpredictable. To address this query, the authors used PC9 lung adenocarcinoma cell lines (hosting the exon 19 (delE746–750) alteration). Cells resistant to the TKIs were generated by treating with increasing concentrations of erlotinib. The resistant cell lines (PC9-ER cells) obtained were characterized with respect to the alterations in the expression of molecules previously reported to confer resistance to the TKIs. To further identify mechanisms associated with EGFR-TKI resistance, quantitative proteomics was used to compare the proteomes of parental PC9 and PC9-ER cells. The proteins from these cells were first separated into soluble and membrane-associated proteins and subsequently fractionated by hydrophilic interaction liquid chromatography (HILIC) to increase the number of proteins identified. The fractions obtained from HILIC were then analysed by liquid chromatography tandem mass spectrometry (LC–MS/MS). A total of 3535 proteins were identified, of which 357 proteins exhibited altered expression and 247 were upregulated and 110 downregulated, in the EGFR-TKI-resistant PC9-ER cells compared to the EGFR-TKI-sensitive, parental PC9 cells. The differentially expressed proteins between EGFR-TKI-sensitive and EGFR-TKI-resistant cells were then classified according to functional subgroups using the bioinformatics platform, Ingenuity Pathway Analysis software. The analysis revealed extensive changes in expression and alterations in several cell processes in the resistant cell lines. A global analysis was performed using selected proteins and the Ingenuity Pathway Analysis

software. This revealed that there was an upregulation of PI3k-AKT-mTOR signalling pathway in EGFR-TKI-resistant cell. The observation was confirmed by analysis of the individual components of the pathway which also indicated that AKT was the key component (Fig. 14.11).

To validate the findings, an additional set of six cell lines, which were resistant to the EGFR-TKI, gefitinib, were generated from the 11–18 NSCLC cell lines. These cell lines harbour the other major EGFR-TKI-sensitizing EGFR mutation, L858R, observed in NSCLC patients. Proteomic analysis of these cells also showed increased activation of the Akt pathway. These resistant cell lines were then cotreated with EGFR-TKI and AKT inhibitors for further confirmation. The observation of AKT being the key molecule which could overcome the resistance arising from different mechanisms was tested in xenografts of the cells in SCID mice. It was noted that TKI and AKT inhibitors could reduce the tumour volume of the PC9-ER cells more effectively than either of them independently, compared to vehicle control. To

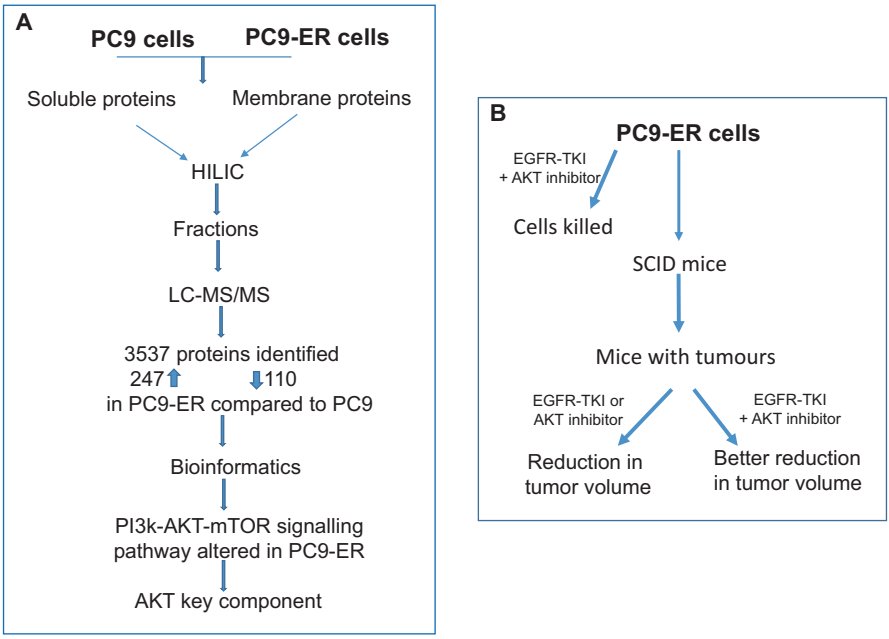

Fig. 14.11 Identification of proteins which may be responsible for drug resistance. (**a**) PC9-NSLC cells and PC9-ER, NSLC cells resistant to erlotinib were separated into soluble and membrane fractions. The proteins in these fractions were separated by hydrophilic interaction chromatography (HILIC). Each of the fractions was subjected to mass spectrometry for protein identification and quantification. Of the 3537 proteins identified, 247 proteins were upregulated and 110 were downregulated in PC9-ER cells compared to PC9 cells. Using bioinformatics, the PI3k-AKT-mTOR signalling pathway was found to be altered in PC9-ER cells, and AKT was identified as the key protein in this pathway. (**b**) The PC9-ER cells were treated with an EGFR-tyrosine kinase inhibitor and AKT inhibitor, and the cells were killed. Tumours were generated in SCID mice with these cells, and when treated with either EGFR-TKI or AKT inhibitor, the reduction in volume of the tumour was less than that when both inhibitors were used together

check if AKT is important in clinical samples, the expression of pAKT was measured by IHC using antibodies to pAKT, in EGFR-mutant NSCLC patients treated with first-line EGFR-TKIs. It was observed that in 60% of tumour samples from patients who had progressed after treatment with EGFR-TKI, there was increased phosphorylation of AKT while only 11% of patients in pretreatment group had increased pAKT.

In summary using proteomic techniques, the importance of AKT in the signalling pathway was determined. This permitted use of combination of an EGFR TKI and an Akt inhibitor for synergistic growth inhibition in vitro and significant growth inhibition in vivo in otherwise EGFR-TKI-resistant NSCLCs harbouring different mechanisms for resistant to TKI. Proteomic tools have therefore provided answers for understanding the mechanisms of resistance and identified a molecule which can be used for prediction of treatment response.

Ligand binding to the EGFR results in activation of a signal cascade in which there is phosphorylation/dephosphorylation of several intracellular molecules. Wang et al. (2017) have investigated the *resistance of NSCLC cells to the TKI gefitinib* by assessing the phospho proteome of NSCLC cells (PC9) which is sensitive and (PC9/gef) which is resistant to gefitinib. PC9 harbours the EGFR exon 19 deletion in the kinase domain, and PC9/gef was derived from it by increasing concentration of gefitinib. To determine if phosphorylation events could contribute to TKI resistance, Western blotting was performed to determine the phosphorylation status of the tyrosines in the kinase domain of EGFR in the two cell lines. There were specific differences indicating that an altered phosphoprotein profile could contribute to resistance to TKI. The protein samples from the two cell lines were subjected to gel-assisted digestion with trypsin. The peptides generated were passed through an IMAC column. The bound phosphopeptides were eluted, and the peptides in the flow through were collected. The phosphopeptides and the peptides in the flow through were subjected to LC-M/MS. The spectral patterns were then analysed using specific software to determine the abundance of the proteins and phosphoproteins in the sensitive and resistant cells. Using in-depth bioinformatics analysis of the data, the authors showed that within the potentially targetable phosphoprotein network, there was increased phosphorylation of the serine threonine kinase CK2 and its substrate HMGA1 which could be attributed to resistance to gefitinib. Knock down of HMGA1 showed that PC9 gefitinib-resistant cells exhibited efficacy to the inhibitor while the same effect was not seen when CK2 was knocked down. These studies have identified HMGA1 for further investigations related to TKI resistance.

Early prognosis of metastatic behaviour of the cancer in patients is much needed. Dunne et al. (2015) have identified Annexin A2 as a protein which is useful for identifying patients who are likely to relapse early with metastatic disease of the prostrate. They have used a combination of proteomic tools for their investigations. FFPE blocks of tissues were taken from 16 men with locally advanced cancer of the prostrate who had been treated with radiotherapy and androgen deprivation. The ten-year follow-up information for disease progression in these patients was available. The time of biochemical failure which is the time from initial randomization

for the trial to the doubling of the level of PSA was taken as the clinical end point for the study. Tissue from the tumour and normal areas in the blocks were dissected and processed for protein extraction. The proteins were then separated either by 1DE or 2DE and the gel pieces cut from the IDE gel, or the protein spots on the 2DE gel were subjected to in-gel digestion with trypsin and identified by MALDI-MS/MS. In a third approach, proteins extracted from the FFPE blocks were digested with trypsin and the peptides separated by reverse phase liquid chromatography. The fractions were then subjected to offline MALDI-MS/MS. Twenty-eight proteins were identified by 2DE-MALDI-MS/MS, 27 by 1DE-MALDI-MS/MS and 242 by LC-MS/MS. These proteins were then compared with data from Human Protein Atlas to determine which of these had been associated with prostate cancer. Twelve proteins were shortlisted from this comparison. Of these PSA, Annexin A2, zinc-alpha-2-glycoprotein (AZGP1, ZAG) and peroxiredoxin-1 (PRDX1) were selected after multiplex Western blotting with antibodies to each.

Another independent set of 16 FFPE blocks was chosen which included patients with different times to relapse. The tumour and normal tissue areas from the FFPE blocks were processed as above and the protein subjected to multiplex Western blotting for the four shortlisted proteins. Analysis of the levels of the proteins showed that high (greater than two- or threefold) relative level of Annexin A2 between the tumour and the control tissue is an independent predictor of early biochemical relapse. Annexin A2 is reported to be present in several tumour cell types and to be associated with tumour cell invasion, migration and adhesion. This proteomic study has identified a protein which can now be used to assess patients who are likely to show early biochemical relapse as assessed by doubling of PSA levels.

It is apparent from the examples cited above that a variety of proteomic tools are now being used to identify the proteins so as to understand and delineate the intricate signalling pathways which are responsible for transformation. Each of the technologies offer unique approaches to address the biological query under investigation. The high-throughput advantages of proteomic tools have indeed contributed immensely towards unravelling the complexities of the cancer signalling pathways.

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Applications of Microarray in Cancer Cell Signaling Pathways

15

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Abstract

Microarrays allow for the analysis of transcription in a large number of genes simultaneously. They have played an important role in the identification of cancer cell signaling pathways. The ease of use and reliability of microarrays has been a result of standardization of assay protocols and consolidation of data analysis techniques. Online public repositories of gene expression profile data have been created, allowing for researchers to test their hypothesis regarding the link between gene signaling and disease. Microarrays have many different applications in the study of cancer. It allows for disease stratification of tumors into different molecular subgroups. They can also provide additional information on disease mechanism, cancer prognosis, and cancer metastasis. Multiple studies have used microarrays on cell lines to perform preclinical screening to predict anticancer drug responses. In addition, they can be used in real-life patient's tissues to provide personalized prediction of drug response.

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Microarray · Gene expression · Signaling pathways · Drug screening · Drug discovery

15.1 Introduction

Cancer development is an evolutionary process, and an accumulation of genetic variations is required to overcome myriads of tightly interconnected regulatory mechanisms. Oncogenic transformations damage usual interlocking of cell signaling systems, and deregulated signaling pathways enable cancer cells to achieve the so-called cancer hallmarks – self-sufficient growth stimulation, resistance to anti-growth signals, apoptosis evasion, uncontrolled replicative potential, heightened angiogenetic pressure, abnormal metabolic pathways, immune surveillance evasion, escalated genomic instability, prolonged inflammatory response, and tissue invasion and metastasis (Hanahan and Weinberg 2000, 2011).

During the last decade, tremendous progress has been made on the therapeutic strategies based on cancer-specific cell signaling phenotypes. Identification of oncogenic signaling pathways underlying those phenotypes can provide important insights on therapeutic options (Vogelstein et al. 2013). However, this requires accurate information on the abundance of subcellular molecules such as DNA or RNA. Microarrays play an important role in providing such information.

Although recent developments in the massive parallel sequencing have overtaken the role of microarrays in many places (Hartmaier et al. 2017; Schram et al. 2017), microarrays still pose a compact unblemished solution for many subcellular molecular investigations.

15.2 Microarrays

First introduced in mid-1990s, DNA microarrays revolutionized the way we monitor subcellular phenomenon (Schena et al. 1995; Lockhart et al. 1996). By arranging cDNA or oligonucleotide probes targeting specific parts of a gene attached to glass slides in regular lattices, they allowed the expansion of genes whose transcriptional activity could be surveyed simultaneously in a sweep of experiment tremendously. Technological development in the field of microarray was so trailblazing that, within a few years, the number of genes on microarrays which had been mere hundreds at initiation soon grew to cover all annotated protein-coding genes in a genome ushering in the era of genome-wide transcriptome analysis even way prior to the completion of whole genome sequence for human. The initial design of microarrays was so elegant and perfect that the overall design was copied to other related platforms allowing for the genome-wide examination of diversified subcellular phenomena beyond gene expression: first to the observation of copy number aberrations (Pinkel et al. 1998) and/or loss of heterozygosity (Lindblad-Toh et al. 2000), microRNA

transcription (Barad et al. 2004; Liu et al. 2004; Sun et al. 2004), alternative splicing (Johnson et al. 2003), etc. In this chapter, however, we will limit our discussion to gene and miRNA arrays for the most part.

Transformation of microarrays as a convenience commodity in molecular biology has been aided substantially not only by the diversification of phenomena that can be screened through them but also by the improvement of trustworthiness in microarray platforms themselves (Consortium et al. 2006; Patterson et al. 2006) as well as ease of use of microarray technology. Confronting mounting skepticism about the consistency of microarray results due to low level of overlap from the same set of RNA samples and the need for a sound regulatory framework to facilitate the adoption of microarrays in pharmacogenomic and toxicogenomic tasks such as drug development, medical diagnostics, and risk assessment in US federal agencies like the US Food and Drug Administration and the US Environmental Protection Agency, a consortium of researchers examined the inter- and intra-platform reproducibility and comparability of gene expression measurements across test sites scattered in multiple locations. In this colossal undertaking of the MicroArray Quality Control (MAQC) project, they concluded high intra-platform consistency across test sites as well as a high level of inter-platform concordance in terms of genes identified as differentially expressed genes (Consortium et al., 2006). The progress in the field of genetics and genomics (Squizzato et al. 2015; Kitts et al. 2016; Tyner et al. 2017) contributed significantly in this gain of confidence on microarray technology since the researchers of MAQC project refined the probe-target gene link by remapping the probe sequences against then completed human genome sequence, throwing away erroneous or ambiguous associations, and consolidating the remaining ones into a "one-probe-to-one-gene" list.

The ease of use for microarrays has been made possible through numerous progresses, mostly by the standardization of assay protocols including intermediate quality assurance assessments and the consolidation of bioinformatics approaches of data processing and analysis. Regardless of specific microarray platforms, biological experiments mainly involve two parts, cDNA library preparation and transcriptome quantification, and the successful standardization of these relied on the tremendous efforts microarray vendors spent to improve the production of quality assured platforms and to establish optimal operational protocols including equipment, reagents, etc. Presently, three commercial vendors mostly provide highly polished platforms of dependable performance to researchers of human as well as several model organisms although design and distributional characteristics of their probes and recommended experimental protocols are clearly different with each other. For instance, Affymetrix (an affiliate of Thermo Fisher Inc.) is well known to use very short-length (~25 nt) probes that are grown on glass slides through photolithographic processing (Lockhart et al. 1996). The obvious limitation due to short probes is compensated by first covering multiple probes, collectively known as probesets in Affymetrix jargon, to target a location of a gene and then by sampling multiple locations of a gene for monitoring its transcriptional activity each targeted with a probeset. Agilent, an affiliate of Hewlett-Packard, one of the most well-known manufacturers of ink-jet printers, offers longer (~60 nt) probes that are

synthesized on glass slides through ink-jet printing method. Longer probes are sufficient to avoid the risk of cross hybridization, and Agilent platforms using longer probes do not require multiple probes for monitoring single gene's expression activity. Illumina, though quite late in entering the transcriptome microarray data market, introduced an ingeniously designed platform based on their renowned BeadArray technology (Fan et al. 2004; Kuhn et al. 2004). By allowing random assembly of beads targeting a specific gene out of a large pool of beads and ensuring all sequences appear in every experimental batch, artifacts due to spatial probe location are minimized, while precision and robustness are gained in expression measurements (Kuhn et al. 2004). These vendors also provide proprietary customized fluidic systems and experimental kits optimized for respective platforms and recommended protocols for guaranteed results.

Algorithmic consensus on the best possible options of experimental design, preprocessing, data analysis of microarray platforms such as statistical inference, (un) supervised classification, and the need for independent validation has been attained for the most part (Allison et al. 2006) although pitfalls and challenges still remain (Tinker et al. 2006). Concerted efforts and responses from research community to polish genomic/genetic annotations and provide open source analysis tools that facilitate novel concepts and ideas in a timely manner have been awe inspiring. Public projects such as R and Bioconductor have been concrete foundation for their circulation and widespread adoption (Team 2018; Gentleman et al. 2004). All of this rendered the job of planning, obtaining, and analyzing microarray data much more modular and straightforward for common platforms these days. However, in this evolution of microarray data analysis, especially in the elucidation of cell signaling pathways/signatures using microarrays, the introduction of gene ontology (GO) and gene sets stands out in their conceptual and practical implication (Ashburner et al. 2000; Mootha et al. 2003). By organizing myriads of biological concepts related to three broad categories, cellular components, biological processes, and molecular functions, in a hierarchical manner and linking genes to each of GO terms, GO made the task of making sense out of differentially expressed genes (DEGs) much more tangible, which has been gracefully extended by releasing the need for preselecting genes of interest such as DEGs through artificial thresholds and consolidating genes into 8 gross collections of curated sets in gene set enrichment analysis (GSEA) later (Subramanian et al. 2005). The advantage of gene sets over GO terms from statistical viewpoint is obvious considering that correction for multiple corrections due to gigantic size of terms of gene sets tested in a sweep is not possible for GO terms due to complex hierarchical dependency paths each term bears from its root terms.

Owing to these developments and the vast amount of gene expression profile data archived in online public repositories such as Gene Expression Omnibus or EBI ArrayExpress (Parkinson et al. 2005; Barrett et al. 2013), or even a curated platform such as Oncomine (Rhodes et al. 2007), researchers and clinicians can easily get the baseline evidence crediting or discrediting the validity of hypothesis concerning links between genes or signaling pathways and diseases. For instance, if we come up with a gene or a signaling pathway that may be involved in multiple

myeloma (MM), we first check if we can see expression difference between MM and normal counterpart for them or if we can see the gene expression or signaling signature is associated with survival in MM using publicly available data (Zhan et al. 2006; Mulligan et al. 2007; Broyl et al. 2010). The advantage of massive amount of publicly available microarray data and gene signature approach has been clearly appreciated when multiple prognostic signatures are combined (Chng et al. 2016). In this study, researchers collected 9 prognostic gene signatures related to multiple myeloma, encompassing gene expression-based prognostic signature (Kuiper et al. 2012; Shaughnessy et al. 2007) and biologically motivated signature like homozygous deletion affected cell death genes (Dickens et al. 2010), chromosomal instability (Chung et al. 2013), and proliferation index (Hose et al. 2011). This study clearly exhibited that by combining multiple gene expression signatures already known to be associated with myeloma prognosis, instead of relying only on a few prognostic signatures, the most optimal combination could be revealed.

15.3 Cell Signaling and Microarrays in Cancers

Although the deregulation of oncogenic signaling pathways has been known important in cancers for a while, unequivocal revelation of the connection between their actual activation and their downstream manifestation requires perturbation of genes and careful monitoring of subsequent expression changes for putative downstream genes, which has been possible only with the help of microarrays (Huang et al. 2003; Lamb et al. 2003; Bild et al. 2006). In a series of seminal works, Nevin's group activated specific oncogenic pathways by employing recombinant adenoviruses for genes like HRAS, MYC, SRC, β-catenin, and E2Fs in otherwise quiescent cells and recorded resulting gene expression cascades across the whole genome for both activated and quiescent cells using microarrays (Huang et al. 2003; Bild et al. 2006). The oncogenic signaling signatures for these genes were then produced by collecting the most discriminant set of genes in an adaptive statistical process involving singular value decomposition and logistic regression (West et al. 2001). One notable aspect of this approach was that the signatures were composed of not only the upregulated genes but also the downregulated genes in the induced cells, which is quite understandable considering the fact that all naturally arising signaling pathways require inhibitory interactions as well as promoting interactions for obvious regulatory purposes. Another was that the notion of signaling pathways was expanded naturally through this approach by retaining genes whose activity was obviously modulated by the activation of oncogenes though not previously known to be direct parts of signaling pathways of interest. Remarkably, these various computational signatures could predict corresponding oncogenic activity of cells in independent datasets. Furthermore, they were able to link activities of these oncogenic signatures to their effectiveness in discerning sample groups of differing risk through their combination in diverse cancer datasets (Bild et al. 2006). Similar technique was also used to uncover the signaling signature of cyclin D1 activation and

its mechanism of action through the mobilization of transcription factor C/EBPβ (Lamb et al. 2003).

Contrary to oncogenes whose artificial activation and the elucidation of internal signaling cascades is relatively straightforward, similar experimental manipulation of tumor suppressor genes (TSGs) and defining gene expression signatures are much more involved and require very specialized recent techniques like CRISPR/Cas (Knott and Doudna 2018) or TALENs (Gaj et al. 2013) system to edit and suppress genes of interest specifically. Unfortunately, these techniques were not available until lately, and the revelation of molecular underpinnings following TSG suppression has lagged behind enormously compared with oncogenic signatures, leaving much of gene signatures of TSGs empty. However, an ingenious proof-of-concept study by Black et al. (2003) was able to catalog the gene signature for inhibiting TSG Rb by sorting out gene expression difference between wild-type and Rb-null mouse embryo fibroblasts as well as cells lacking other Rb family members.

Despite the benefit of the phenomenal experiments of activation oncogenes/deactivation TSGs and recording signaling cascades, this approach requires lots of resources, efforts and specialized setups, limiting its wide adoption. As a complementary strategy, computationally constructed signatures utilizing tens of thousands of gene expression profiles came to be more prevalent while the use of microarray as a means of genome-wide molecular scrutiny has been immensely expanded (Segal et al. 2003, 2004). This approach had natural appeal that gene expression signatures could be produced en masse based solely on expression profiles without resorting to any biological information, allowing for unbiased characterization of molecular phenotypes represented in gene expression data. In a series of pioneering work, Segal et al. introduced the concept of "module map" where molecular activities shared by groups of genes across diverse phenotypic conditions and experiments were summarized in "modules" whose patterns of induction and repression could represent specific conditions or underlying disease mechanisms. Using wide sets of gene expression data of a model organism *S. cerevisiae* under diverse environmental perturbations and knowledge on candidate regulators such as transcription factors and signal transduction molecules, they first proved conclusively that the concept of gene modules was not fictitious being produced out of computational artifacts but stemmed solidly from underlying regulatory mechanisms with concrete set of regulatory modules mobilized by diverse experimental conditions. Most of the computationally recovered gene modules turned out to include genes known to be regulated by the modules' predicted regulators, and many had a match between a predicted regulator and its known *cis*-regulatory binding motif, further consolidating the concept of gene module (Segal et al. 2003). For the case of human where specific conditional perturbation information was impossible to obtain, they resorted to diverse disease datasets and gene sets, groups of genes from numerous sources collected under the same biological contexts such as biological pathways, tissue-specific genes, and gene ontology. Noting that not all genes in a gene set participate in the same gene module represented by a harmonized expression pattern, they were further refined by clustering expression profiles from several gene sets of similar

expression pattern. This portrayal was particularly useful in identifying biological mechanisms for specific subtypes of patient as illustrated for growth inhibitory module in acute leukemia or the steroid catabolism module in hepatocellular carcinoma (HCC) or the bone osteoblastic module in a subset of breast cancer, a subset of non-tumor hepatitis-infected liver, and HCC (Segal et al. 2004).

The two approaches discussed in this section depict diametrical tactics of clarifying cell signaling using microarrays in biological systems, and they possess respective merits and demerits. Although biologically validated oncogenic signatures of the first approach have clear association with specific molecular mechanisms and can be very useful especially in clinics, they are too few to be exploited routinely. To the contrary, although computationally composed signatures of the second approach are abundant and rich in context for regular employment in principle, their biological implications are often quite complicated and ambiguous in practice. An intermediate amalgamation between these two approaches often provides quite reliable results (Ramaswamy et al. 2003; Rhodes et al. 2004; Nguyen et al. 2009; Evans et al. 2016; Ben-Shoshan et al. 2017; Jung et al. 2017; Scott et al. 2017; Chung et al. 2013; Yan et al. 2016; Selvarajan et al. 2017). In one approach, bioclinical information of specific context was provided from the onset, genes whose expression activity was highly correlated or anticorrelated with the information were selected, and, finally, signal activities captured in multigene signatures were numerically summarized. The bioclinical information addressed in this context is diverse: for instance, metastasis signature was constructed by comparing primary tumors and metastases (Ramaswamy et al. 2003); proliferation signature was constructed by aligning gene expression profiles to immunohistochemically defined proliferation activity (Scott et al. 2017); chromosomal instability signature was constructed by comparing samples of high number of copy number aberrations with those of low number of copy number aberrations (Chung et al. 2013). However, for cases where potential genes of interest are already known as in the case of signaling pathways or known biological processes, researchers can proceed in a more targeted manner (Nguyen et al. 2009; Yan et al. 2016; Ben-Shoshan et al. 2017; Jung et al. 2017; Selvarajan et al. 2017). In this case, since core groups of genes are already known from the start, microarray data can be utilized to filter out genes whose activity profiles do not fit into the pattern displayed by the core groups as shown for WNT signaling in lung adenocarcinoma (Nguyen et al. 2009), Myc activity in various cancers (Jung et al. 2017), NFκB signaling in liver inflammation (Ben-Shoshan et al. 2017), JAK-STAT signaling (Yan et al. 2016) and Myc signaling (Selvarajan et al. 2017) in NK/T-cell lymphoma, etc. By combining best of two extreme approaches, the potential of this intermediate scheme in practical applications is virtually endless, only limited by researchers' creativity.

Recent developments in drug discovery have expanded the repertoire of cancer drugs with clear target signaling pathways, and activity patterns of validated oncogenic signatures can direct choices of optimized therapy for clinicians (Nelson et al. 2017; Fruman et al. 2017; Joerger and Fersht 2016; Ribas and Wolchok 2013). However, the spectrum of biologically validated oncogenic signatures is too limited to be useful presently and should be expanded drastically for better adoption in the

future. Due to the abundance of members and clarity of construction, to the contrary, computationally constructed signatures introduced in this section are expected to be more useful in dissecting molecular complexity of phenotypes and reconstructing them into coherent gene groups. However, since it is hard to associate many of the gene groups to specific molecular mechanisms of signaling pathways in many cases and single gene group is composed of genes known to be ascribed to numerous molecular mechanisms in other cases, the biological interpretation of this reconstruction is ambiguous.

15.4 Potential Utility of Microarrays in Cancers

Conventional wisdom of mining microarray data is roughly explained from two subgroups: unsupervised or supervised approaches (Butte 2002). In unsupervised analyses, researchers don't assume any a priori knowledge on sample phenotypes and try to uncover signals hidden in molecular activity profiles. In supervised analyses, however, researchers separate samples by their given phenotypes and try to see which molecular profiles are implicated between the phenotypic classes or can be utilized to differentiate future samples of similar phenotypes. Even for the unsupervised analysis cases, researchers can further adopt supervised analysis techniques afterward when signals hidden in molecular activity profiles are successfully uncovered and distinct sample groups can be produced from the signals.

15.4.1 Disease Stratification

Despite seemingly of limited utility, unsupervised analyses of microarrays have produced myriads of phenomenal results especially in stratification of cancers unveiling intrinsic intra-tumor heterogeneity in virtually all cancers analyzed using microarrays shattering the myths that all tumors of an anatomical tissue are the same and raising the need for redefine definitive baseline groupings based on molecular phenotypes. One archetypal example is the 5 intrinsic subtypes of breast cancer, luminal A, luminal B, HER2-enriched, basal-like, and normal-like subtypes, revealed through microarray data analyses (Sorlie et al. 2001; Perou et al. 2000). These intrinsic subtypes based on distinct molecular programs not only corresponded well with immunohistochemistry-defined subtypes but also exhibited distinct groups of patient outcome.

Furthermore, the 5 intrinsic subtypes defined by using gene-by-gene expression profiles could be nicely represented by oncogenic signaling signature indices with particular patterns of signaling activity, and the signaling pathway activity profiles exhibited superb predictive power for sample classification as well in an independent study (Gatza et al. 2010). Similar gene expression-based patient stratification has been illustrated repeatedly. Another interesting example is the translocation/cyclin D expression (TC) classification of multiple myeloma based on the dysregulation of oncogenes due to recurrent Ig translocations and increased expression of

cyclins D1 and/or D2 compared with normal bone marrow plasma cells (Bergsagel et al. 2005). Since TC classification is based on dysregulation of oncogenes, classes in this scheme are expected to depict robust underlying molecular mechanisms and hence can be observed in other studies again, which actually happened (Zhan et al. 2006; Broyl et al. 2010).

15.4.2 Disease Mechanism

To the contrary, the possibility of supervised microarray data utilization is remarkably flexible only limited by researchers' creativity as has been already witnessed in numerous signatures in this review. This type of usage has been extremely useful especially when one is interested in the so-called molecular biomarkers, and some classes of molecular biomarkers deserve special mention as concrete applications of microarrays. In utilizing microarrays for clinical cause, several issues other than disease stratification have also been scrutinized. The most fundamental was of course the disease mechanism, and researchers' interests have widened to the transcriptome analysis beyond mRNAs recently often aiming at non-coding RNAs such as miRNAs or lncRNAs.

15.4.3 Cancer Prognosis

Cancer prognostic markers linking groups of genes to patients' overall survival or disease recurrence are a perennial topic of microarray, and researchers used microarrays to uncover diverse biomarkers that can tell patients of higher risk of early death or recurrence after therapy so many times so far (Zhang et al. 2018; Barter et al. 2014; Ascierto et al. 2012; Wang et al. 2016; Han et al. 2012). Prognostic gene expression signatures are in general of two types, those constructed with patient prognostic outcome in mind without any other consideration (Zhang et al. 2018; Wang et al. 2016; Barter et al. 2014; Han et al. 2012) and those that are relevant to specific biological context which turned out to be prognostically relevant as well later (Ascierto et al. 2012; Chung et al. 2013; Hose et al. 2011; Dickens et al. 2010). When prognostic outcome was of primary objective, researchers usually applied Cox regression to overall survival or progression free survival and selected genes whose p-values were smaller than some fixed cutoff. However, those could be also constructed by comparing patients of early event with those of late event. Then, the signatures could be further refined through rounds of filtering using biological process or pathway information. The procedural simplicity is the main benefit of this type of approach although the signatures themselves may not offer biological insight regarding disease etiology or possible choice of therapeutic strategy. To the contrary, there is no guarantee that biological context-guided signatures could also be prognostically related. However, once they are related, they pose greater chance of therapeutically tractable.

15.4.4 Cancer Metastasis

Cancer metastasis, the most important cause of cancer deaths, has been examined continually and produced several gene and miRNA signatures (Wotschofsky et al. 2016; Qi et al. 2016; Das et al. 2016; Yau et al. 2013; Daves et al. 2011; Yau et al. 2010). For instance, Davies et al. combined 18 publicly available microarray datasets comparing distant metastases with primary tumors from several solid cancers and looked for a common metastasis signature (Daves et al. 2011). After collecting 79 genes for common metastasis signature, mostly downregulated except one and enriched in many pathways previously implicated in metastasis, such as integrin signaling, calcium signaling, and VEGF signaling, they validated the independent 6 datasets confirming that the signature was significantly implicated when metastases and primary tumors were compared. This does not mean that no more novel target genes specific for oncogenic signaling pathway could be found for solid tumors as evidenced in Qi et al. where three new genes targeted by Wnt/β-catenin signaling pathway were identified from the colorectal cancers through the combination of microarray and complementary molecular assays (Qi et al. 2016). The search for metastatic signature in hormone receptor-negative and triple-negative breast cancer (TNBC) patients poses an interesting evolutionary process (Das et al. 2016; Yau et al. 2010, 2013). With the help of three public mRNA microarray datasets, researchers first built a 14-gene metastasis signature and validated it with other three independent datasets first (Yau et al. 2010). Later, they narrowed the signature to 5-gene signature to make it suitable for an RT-PCR assay platform designed for FFPE tumor samples (Yau et al. 2013). Then, an independent group proposed a miRNA, miR-720, as a downstream target of ADAM8-induced ERK signaling cascade that promotes migratory and invasive phenotype in TNBC.

15.4.5 Preclinical Drug Screening

Microarrays have been indispensable tool for anticancer drug-related studies. In particular, efforts to utilize preclinical screening information in predicting actual patient's drug response go all the way back to the early days of microarray where Weinstein group of US National Cancer Institute tried to combine a pattern of GI_{50} of ~60 K anticancer drugs over a panel of preselected 60 cell lines and molecular profiles over the same cell lines through an informatic way to link anticancer drugs to molecular targets or genes (Scherf et al. 2000; Weinstein et al. 1997). This approach attracted much attention since it decoupled the complicated task of associating drugs and genes into two relatively well-established routine tasks: linking drugs and cell lines, which was accomplished by assessing GI_{50} to each of drug-cell line combination, and genes and cell lines, which was accomplished by simple molecular profiling of cell lines. Two research groups tackled the same problem from a slightly altered rationale later and produced two pharmacogenomics databases (GDC and CCLE, respectively) independently (Garnett et al. 2012; Barretina et al. 2012). Rather than focusing on connecting molecular features to drug efficacy directly, they concentrated on characterizing genetic changes of much widened

number of cell lines by using sequencing and genome-wide copy number aberration (CNA) investigation and tried to connect those genetic changes to anticancer drug efficacy, hoping to uncover anticancer drug sensitivity markers from them for a small number of selected drugs. GDC and CCLE were different only on specific details like the panel cell lines, drugs, drug efficacy assessment, and sequencing/CNA platforms. Intriguingly, however, despite their similar rationale and methodology, confronting reports on the congruence or incongruence between the two results appeared later. First, Haibe-Kains et al. argued that, while the two results manifested high level of concordance on gene expression profiles and genetic changes, drug response data were highly discordant causing serious concerns about the utility of the two massive gene-drug association data (Haibe-Kains et al. 2013). Later, however, the two groups of original studies joined forces together and reported that their pharmacological data exhibited reasonable consistency when biologically grounded analytical considerations were incorporated and, most importantly, data from either study yielded similar predictors of drug response (Cancer Cell Line Encyclopedia and Genomics of Drug Sensitivity in Cancer 2015).

"Connectivity map" (CMap) developed by researchers of Broad Institute (other than those involved in CCLE) was of a different vein: contrary to the efforts like NCI60 profiling or GDS/CCLE where the characterization of cell lines used for preclinical screening was of primary goal, CMap team wanted a generic methodology of predicting drug sensitivity by connecting diseases, physiological processes, and small molecule therapeutics (Lamb et al. 2006). Here, they treated 164 small molecules in first version which expanded to over thousand compounds in later version mostly to a cell line, MCF7, at a fixed concentration 10 μM and compared the gene expression profile against a standard control. When new drug profile was obtained, then, the profile was compared with existing small molecule profiles similar to the way of GSEA. The connectivity score would record if up-/downregulated genes of new drug profile appeared near the top-positive/bottom-negative DEGs of an existing drug profile or if they appear at random positions. Despite its limited span of experimental conditions and small molecules, connectivity map is still a popular tool for researchers interested in drug mechanism (Schnell et al. 2015; Churchman et al. 2015), and greatly expanded system by targeting the expression pattern of a reduced number of transcripts perturbed with a substantially expanded series of small molecules has been introduced quite recently (Subramanian et al. 2017). Furthermore, CMap became an integral tool for drug repositioning, repurposing already known drugs to new therapeutic assignments, because it allowed the identification of putative therapeutic drugs by the usual practice of utilizing microarrays for the cultivation of "disease signatures" (Koudijs et al. 2018; De Bastiani et al. 2018; Hurle et al. 2013).

15.4.6 Drug Response/Resistance Prediction

Predicting drug response or resistance from preclinical screening database, admitting its great potential, cannot be accurate since experimental conditions for preclinical screening cannot faithfully replicate the real-life conditions cancer cells

experience inside a patient's body, and promising results or disappointing ones from preclinical screening do not guarantee such results in real trial in principle. Naturally researchers believe obtaining molecular signatures from specimens of actual patients' tissues can be more trustworthy, leading to more genuine biomarkers for drug response/resistance prediction. The need for better drug response/resistance markers is clearly understandable considering that anticancer drugs can produce great results when used at right patients as has been witnessed for the case of imatinib in chronic myeloid leukemia or cisplatin-based chemotherapy in testicular cancer but develops resistance in wrong patients (Gambacorti-Passerini and Piazza 2015; Hanna and Einhorn 2014; Tada et al. 2011; Burguillo et al. 2010). In predicting individuals who would get benefit or not from anticancer drug treatment, microarrays have been a critical medium (Cha et al. 2016; Tian et al. 2013; Burington et al. 2011; Artemov et al. 2015; Wang et al. 2011, 2014; Zhou et al. 2018; Chen et al. 2018; Wu et al. 2014). Interestingly, regardless of whether researchers claimed their signatures as response signature or resistance signature, their methodological strategies were fundamentally similar, comparison of samples responding to the drugs with those non-responding, and the signatures encompassed gene signatures as well as miRNA signatures.

15.5 Conclusion

Although the signature summarization was not standardized and diversified estimation methodologies were employed, they all share similar attributes. Microarray has been useful in interrogating cell signaling in cancer, and these types of interrogation may provide useful functional context to other genomic studies such as DNA mutations by NGS or miRNA expression. It is also useful to study prognosis and biology and provide a platform for drug screening and discovery, especially the potential for repurposing of approved drugs.

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Nanotechnology: The Future for Cancer Treatment

16

Yogita Patil-Sen, Ashwin Narain, Simran Asawa, and Tanvi Tavarna

Abstract

Nanotechnology, which is defined as the science behind the structures within the size range of 1–100 nm, potentially holds the key to treat several chronic diseases such as cardiovascular diseases, respiratory diseases and cancer. Nanoscale structures can provide promising tools for various applications in nanomedicine including those in drug delivery of therapeutics and imaging. Present-day cancer treatments suffer from severe side effects and lack specificity, thus affecting healthy cells. Nanoparticles, however, can preferentially accumulate only at the tumour site or can be targeted to cancer cells by surface functionalization using ligands. A major advantage of nanoparticles lies in the scope of surface modification and encapsulation of poorly soluble anticancer drug. This translates into higher therapeutic efficacy and lower toxicity for nanoparticle therapeutics. Thus, nanoparticles offer myriad potential in medical science. This chapter highlights various types of nanoparticles and targeting moieties that have potential to serve as drug carriers that can selectively target tumour cells.

Keywords

Nanoparticles · Targeted cancer therapy · Drug delivery · Targeting ligands · Moieties · Therapeutic

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16.1 Introduction

Cancer is the second leading cause of mortality, after cardiovascular diseases, and according to the World Health Organization (WHO), cancer accounted for 8.8 million deaths in 2015 (http://www.who.int/cancer/en/, https://www.cancer.gov/about-cancer/understanding/what-is-cancer) (1, 2). There are more than 100 types of cancer which are usually named for the organs or tissues where the cancer forms. Lung, liver and prostate cancers are the most common types of cancers in men, while breast and cervical cancers are the most common in women. Cancer is a generic term for a group of disease in which cells divide in an uncontrolled manner and can invade surrounding parts of the body and thus can spread to other organs. Cancer cells grow into a mass called tumour and causes destruction of healthy tissues around it. Mutation in the genetic material of the cell due to environmental or inherited factors and an unhealthy lifestyle are the main causes of cancer. Radiotherapy and surgery are the most effective anticancer treatments for non-metastatic and local cancers, but these treatments are inefficient when cancer has spread throughout the body. Chemotherapy, which involves injecting cytotoxic compound/drug into the body, is the third option for cancer treatment. For metastatic cancers, chemotherapy is the choice of treatment as the drugs can reach body organs through the bloodstream. Unfortunately, all the current cancer therapies are non-specific and can damage the healthy cells along with the cancerous cells causing toxicity and undesirable side effects (Fahs et al. 2015). Therefore, a better understanding of biological interactions of therapeutic drugs on a cellular level and the design and development of novel drugs may help to circumvent the issues associated with the current therapies (Fahs et al. 2015; Fahs et al. 2014). Immunotherapy, gene therapy, laser treatment and hyperthermia are some of the therapies that are recently explored (Borghaei et al. 2009; Rosenberg 1991; Cross and Burmester 2006; Majid et al. 2017; Patil-Sen and Chhabria 2018). Moreover, targeted therapies which will reduce the adverse effects and hence the morbidity have also been extensively researched over the past several years.

16.2 Nanotechnology for Cancer Therapy

Nanomedicine, a branch of nanotechnology, provides a tool to manufacture and engineer materials to treat diseases including cancer. Nanomedicine deals with systems and devices, e.g. nanoparticles, whose size range is between 1 and 100 nm. Nanoparticles preferentially accumulate only at the tumour site. This is because of the enhanced permeability and retention (EPR) effect (Matsumura and Maeda 1986). Thus, it not only affects the favourable bio-distribution of the nanoparticles at the target site and subsequent higher efficacy but also helps in overcoming the higher toxicity of bare drugs due to lack of specificity.

Major advantages of nanoparticles over the traditional chemotherapy lie in their (i) ultra-small size which improves their blood circulation and tissue distribution, thus assisting their intracellular uptake; (ii) ability to encapsulate both hydrophilic and hydrophobic drugs, thus improving solubility of drugs; and (iii) scope for

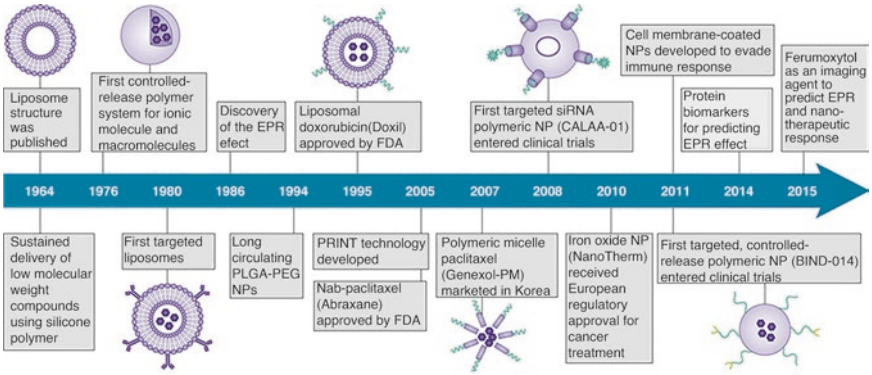

Fig. 16.1 Major developments in the field of nanomedicine for cancer therapy. NP, nanoparticle; PRINT, particle replication in non-wetting template; siRNA, small interfering RNA. (Adapted with permission from Shi et al. 2017)

surface modification with ligands for targeted therapy (Bertrand et al. 2014; Xu et al. 2015; Jin et al. 2014; Brannon-Peppas and Blanchette 2012; Steichen et al. 2013; Peer et al. 2007). Moreover, their large surface to volume ratio, biodegradability and low toxicity make the nanoparticles excellent choice for various biomedical applications (Bertrand and Leroux 2012).

One of the most explored applications of nanoparticles lies in the area of advanced drug delivery due to their varied composition, morphology and surface characteristics (Liechty and Peppas 2012; Estanqueiro et al. 2015; Steichen et al. 2013; Peer et al. 2007). Since the early ages of nanotherapeutic research, there has been a wide range of nanoparticle compositions and structures that are extensively studied. The most common architectures for targeted drug delivery applications include liposomes, micelles, dendrimers, polymeric nanoparticles, metallic nanoparticles and cell-membrane-coated nanoparticles (Wang et al. 2012a; Majid et al. 2017; Kulkarni et al. 2015a, b; Patil-Sen et al. 2016; Gaunt et al. 2015; Narain et al. 2017; Patil-Sen and Chhabria 2018). These systems can further be modified by the addition of different biomolecules or pH, magnetic, temperature or photosensitive vectors (Holgado et al. 2012; Liu and Zhang 2012; Bibi et al. 2012; Delyagina et al. 2011). Currently, there are a very limited number of nanoparticles that have made it from bench to market translation and hence the interest in developing novel nanomedicines (Dawidczyk et al. 2014; Zhang et al. 2008; Barenholz 2012). Figure 16.1 indicates the major developments in the field of nanomedicine for cancer therapy.

16.3 Types of Nanoparticles

Various types of nanoparticles have been explored in the past several years because of their suitability in biomedical applications. These are discussed in detail in the following subsections. Some of these nanoparticle types are represented in Fig. 16.2.

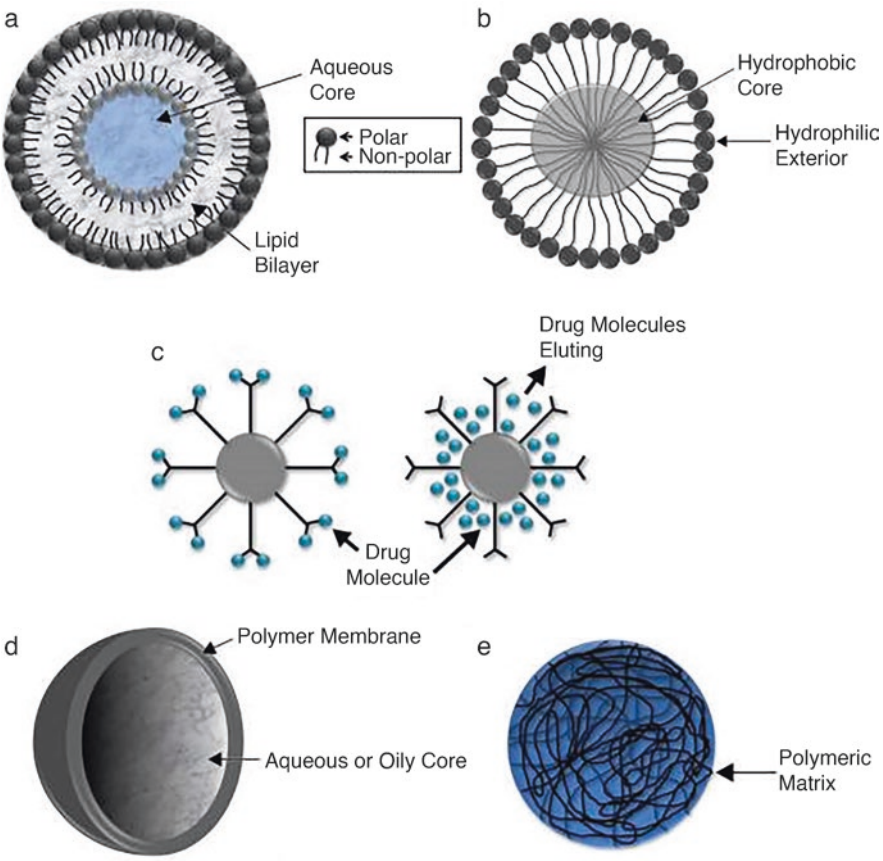

Fig. 16.2 **Schematic presentation of nanoparticles**. (**a**) Liposome, (**b**) micelle, (**c**) dendrimers functionalized with complexed (left) and encapsulated (right) drug molecules, (**d**) nano-sphere, and (**e**) nano-capsule. (Reproduced with permission from Steichen et al. 2013)

16.3.1 Liposomes

Liposomes are one of the oldest nanoparticles to be used in medicine. Liposomes are formed by amphiphilic molecules such as phospholipids which consist of a polar head group and a non-polar tail group. Amphiphilic molecules self-assemble in the presence of water to form various nanostructures in which the hydrophilic head group is in contact with water and the hydrophobic tail group is away from water. Lipids which are an integral part of the biological membranes exist as bilayered lamellar nanostructure (Patil-Sen et al. 2004). Liposomes are spherical structures with an aqueous core and vesicle shell which can be made of single or multiple bilayered membranes. Due to their structural and compositional similarity with the cell membrane, liposomes can be referred to as bioinspired nanoplatforms (Hafner et al. 2014). Depending upon their lamellarity, liposomes can form uni-, oligo- or

multi-lamellar vesicles. The size of liposomes can thus range from tens of nanometres to few micrometres. Unilamellar vesicles which consist of one lipid bilayer are 50–200 nm in diameter, whereas multilamellar vesicles comprising several concentric lipid bilayers have diameters of 1–5 μm.

Liposomes have unique ability to encapsulate both hydrophilic and hydrophobic therapeutic in their structure. The hydrophilic drug can be encapsulated into aqueous core, and hydrophobic drug can be encapsulated into hydrophobic chains. Thus, liposomes serve as excellent nanocarriers for drug delivery applications. However, because of the small space available in the hydrophobic region, the loading capacity of poorly soluble drugs is limited (Malam et al. 2009). The other difficulty with liposomes is that there is limited control over the drug release profile. Recent developments of thermo-, pH-sensitive and ultrasound-triggered drug release strategies do provide improvements (Kono et al. 2010; Simões et al. 2004; Schroeder et al. 2009; May and Li 2013).

Although liposomes are biocompatible and biodegradable nanostructures, however, owing to their small size these are rapidly captured by the mononuclear phagocyte system (MPS) and discarded from blood circulation. The stability and circulation half-life time of the liposomes can be increased by functionalizing with polymers such as polyethylene glycol (PEG) (Torchilin 2005). Further, the possibility of surface modification using biomolecules as ligands for active and passive targeting increases the specificity and biocompatibility (Haley and Frenkel 2008). Liposomes can be used as carriers of drug for active targeting by binding antibody fragments or monoclonal antibodies to the surface of liposomes, called immunoliposomes, thus enhancing the antitumour activity of the drug and reducing the systematic toxicity (Carter 2001; Torchilin 2005; Kontermann 2006). These immunoliposomes allow much higher amounts of cytotoxic drugs than antibody-drug conjugates that can only be coupled with few molar equivalents of drugs (Carter 2001). Moreover, multiple antibodies and other targeting moieties can be incorporated into immunoliposomes which can increase the targeting activity of antibodies (Allen 2002).

New strategies such as use of the combination of different therapeutic agents in liposomal formulation have recently gathered interest. Various liposomal formulations are currently under clinical trials: (I) CPX-351, a liposomal formulation containing cytarabine and daunorubicin for the treatment of acute myeloid leukaemia (Feldman et al. 2012); (II) CPX-1, a liposomal formulation containing irinotecan hydrochloride and floxuridine for the treatment of colorectal cancer (Batist et al. 2009; Fan and Zhang 2013); and (III) ThermoDox, a thermo-responsive liposome containing doxorubicin for the treatment of hepatocellular carcinoma (Wood et al. 2012).

Liposomes are also used in gene cancer therapy for delivery of synthetic short interfering RNA (siRNA) for silencing tumour genes which are involved in cancer advancement or plasmid encoding cDNAs for expression of tumour suppressors. Coupled with active targeting providing specificity, the nucleic acids are also protected by macrophage uptake due to liposomes (Hughes et al. 2010). There are a number of siRNA-loaded liposomal formulations under clinical trials, e.g. TKM-PLK1 for the treatment of neuroendocrine tumours and adrenocortical carcinoma (Burnett et al. 2011).

Thus, the pharmacokinetics and bio-distribution of a drug can be improved using liposome drug formulations by exhibiting longer circulation time in blood. It is easy to modify the physicochemical properties of liposomes such as size, shape and surface charge by altering the lipid composition and surface modification agents. Many promising clinical studies are under development to increase the antitumour activity of the cytotoxic drugs and to reduce the toxicity. Despite so many advantages of liposomal nanocarriers, only 5 liposome-based systems have been approved by Food and Drug Administration (FDA) (Fan and Zhang 2013). Doxorubicin HCl stealth liposome injection (Doxil) was the first liposomal carrier approved by FDA (Northfelt et al. 1998). No immunoliposome formulation has yet reached the FDA approval; however, many preclinical studies are reported in the literature and a few clinical trials are in progress (Drummond et al. 2010; Park et al. 2002).

16.3.2 Micelle

Micelles are self-assembled spherical nanostructures made up of amphiphilic molecules with a hydrophobic core and hydrophilic exterior (Liechty and Peppas 2012). The typical size of micellar nanostructures is less than 100 nm, which inhibits their uptake by the MPS/reticuloendothelial system (RES). The hydrophobic core can solubilize poorly water soluble drugs, and the hydrophilic exterior protects the hydrophobic drugs loaded in the core. This results in long circulation times of the drug in the bloodstream (Torchilin 2007). Thus, micelles are promising carrier system for delivery of cytotoxic drugs which have limited solubility in water.

Presently, there are a few micellar drug formulations that are in the clinical trials for anticancer therapies, e.g. Genexol-PM (paclitaxel encapsulated in monomethoxy-poly(ethylene glycol)-block-poly(D,L-lactide)), which is being used against metastatic breast cancer, urothelial cancer, head and neck cancer, non-small cell lung cancer, ovarian cancer and metastatic pancreatic cancer (Lee et al. 2008). BIND-014 is docetaxel-loaded PEG-poly(lactic-co-glycolic acid) (PLGA) micelles to treat prostate cancer (Bobo et al. 2016), Paclical® poliglumex, paclitaxel-loaded poly-(L-glutamic acid) polymeric micelle to treat ovarian cancer (Khanna et al. 2015), and NC-6004 (Nanoplain) consists of cisplatin-loaded PEG + poly-glutamic acid micelles to treat solid tumours (Plummer et al. 2011).

Although there are encouraging reports about the use of micelles in clinical trial stage, there are some challenges with these systems which need to be overcome. Inadequate stability during circulation in the bloodstream can cause untimely drug release which may lead to major side effects and subsequently affect the efficacy (Torchilin 2007). Further, the problem of targeting the micelles to the specific tissue poses a great challenge. Surface functionalization using biomolecules and stimuli-responsive drug release are some of the strategies that have been researched to overcome the aforementioned issues (Bae et al. 2005; Du et al. 2010; Rapoport 2007).

16.3.3 Dendrimers

Dendrimers are well-defined, radially branched, symmetric, nanostructured three-dimensional macromolecules synthesized from natural or synthetic chemicals such as amino acids, nucleotides and sugars (Baker Jr 2009). These are made of repeated branches of dendron containing a single chemical group, called a focal point. Dendrimer chemistry has its own nomenclature system, and the term "generation" in dendrimer nomenclature represents the repeating monomer units, i.e. the number of branching points from the centre of the dendrimer towards the periphery. The centre or the core part of the dendrimer is denoted as G0, and a dendrimer having four focal points when going from the centre to the periphery is called a fourth-generation dendrimer, G4.

Typically, dendrimers are 10–100 nm in size and consist of a central core of initiator, surrounded by repeated branching units and terminal functional groups (Medina and El-Sayed 2009). Moreover, these globular nanostructures contain inner cavities which are formed due to hyperbranched architecture from the central core. The inner cavities can be exploited for the encapsulation of active ingredients such as drug molecules. This three-dimensional tree-like structure not only allows high loading of drug molecule but also protects it from its outside environment, increasing the stability of the active compound and thus prolonging the residence time of the drug in the circulatory system (Majoros et al. 2006). Encapsulation of drug molecules into dendrimer structures is based on various mechanisms including electrostatic, hydrophobic, H-bonding and acid–base effects in the interior of the dendrimer. Besides, drug molecules might also be covalently conjugated to the dendrimer particle.

Literature suggests the use of dendrimers in both active and passive targeting for anticancer therapy. Antibody–dendrimer conjugates, peptide–dendrimer conjugates and folic-acid-functionalized dendrimers have been reported for active targeting and have shown better efficacy compared to free drug or untargeted controls (Thomas et al. 2004; Dijkgraaf et al. 2007; Shi 2013). There are several examples presenting the use of dendrimers as carriers for passive targeting, for example, conjugation of sodium carboxy-terminated G-3.5 poly(amidoamine) (PAMAM) dendrimer with cisplatin (Wang et al. 2013; Malik et al. 1999; Kojima et al. 2000). Moreover, dendrimers can be multifunctionalized by incorporating a therapeutic agent and imaging agent, and thus these can be employed in theranostic applications (Menjoge et al. 2010).

Even after so many reports indicating a tremendous potential of dendrimers in medicine, these nanocarriers face several challenges, a major being toxicity of these nanoparticles (Duncan and Izzo 2005). Further, as the release of the drug is by depolymerization of the dendrimer, it sometimes leads to an uncontrolled release of the drug. Thus, research needs to be focused on the areas to address these issues.

16.3.4 Polymeric Nanoparticles

Polymeric nanoparticles are nanosized spherical colloidal systems made of biodegradable polymers. These nanoparticles are formulated by a self-assembly of two or more block-copolymers which differ in their hydrophilicity. The copolymers self-assemble in such a way that the hydrophobic blocks form the core to minimize their exposure to aqueous environment, whereas the hydrophilic blocks stabilize the core by forming a shell (Farokhzad et al. 2004b). Thus, polymeric nanoparticles can be used for delivery of both hydrophobic and hydrophilic drugs as well as other macromolecules such as genes and peptides (Kamaly et al. 2012). Further, the surface functionalization and simple conjugation methods allow polymeric nanoparticles to carry multiple ligands and imaging molecules which can be used for cancer diagnostics and targeting and in imaging (Duncan 2006).

Many synthetic polymers such as PLGA, polyglycolic acid (PGA), polyethylene glycol (PEG), N-(2-hydroxypropyl)-methacrylamide copolymer (HPMA) and polylactic acid (PLA) have been extensively studied in translational medicine due to their biocompatible and biodegradable properties (Sah et al. 2013). Moreover, natural polymers such as alginate, dextran and chitosan have also been explored in nanomedicine due to their natural abundance, in addition to their biocompatible and biodegradable characteristics. However, compared to the synthetic polymers which produce sustained release of therapeutics, natural polymers have been reported to produce fast drug release (Liu et al. 2008).

Some of the examples of polymeric nanoparticles being used for drug delivery are as follows: (I) Abraxane, an albumin-bound paclitaxel nanoparticle, has been approved by the FDA for metastatic breast cancer, first-line treatment of non-small-cell lung cancer and metastatic pancreatic cancer; other clinical trials are under progress; (II) Paclical® poliglumex, a paclitaxel-encapsulated PLGA nanoparticle formulation has shown to have a stronger cytotoxic effects on cancer cells in vitro and in vivo, compared to the commercial formulation of paclitaxel alone, Taxol (Danhier et al. 2009); and (III) cationic lipid-coated PLGA nanoparticles have also been reported for siRNA delivery (Hasan et al. 2012). Despite their promising properties, several polymeric nanoparticles are currently in clinical trials and face a challenge with RES removal and, thus, have not yet been approved by the FDA (Fadeel 2012).

16.3.5 Carbon Nanotubes

Carbon nanotubes are a network of carbon atoms shaped into hollow cylindrical tubes. These nanostructures are synthesized by an arc discharge or chemical vapour deposition of graphite (Mallick and Strydom 2013). The size of carbon nanotubes is around 1–4 nm in diameter and 1–100 μm in length (Sinha and Yeow 2005). Insolubility of carbon nanotubes in all solvents poses issues concerning their

toxicity. However, by controlling the length and diameter of the nanotubes and modifying their chemical structures, their water solubility can be enhanced and biocompatibility can be increased and their toxicity can be decreased (Bianco et al. 2011). An anticancer drug can be loaded on the surface of the carbon nanotubes or in their inner cavity (Ajima et al. 2008; Wu et al. 2009). These nanocarriers are capable of crossing the plasma membrane by endocytosis or needle-like penetration, and their tumour cell specificity can be improved by functionalizing the nanotubes which can bind to targeting moieties (Zhang et al. 2009; Fabbro et al. 2012).

Although there are no FDA approvals or clinical trials in process up to date using carbon nanotubes, in vitro and in vivo preclinical studies suggest that these are promising systems for passive targeting in cancer treatment (Fabbro et al. 2012). Few examples of these studies are listed here. Cisplatin and a targeting moiety, epidermal growth factor EGF, conjugated to single-walled carbon nanotubes, have shown improved efficacy against squamous cancer cells which overexpress the epidermal growth factor receptor, EGFR. Compared with the untargeted carbon nanotubes without EGF molecules, those conjugated with EGF showed increased accumulation and uptake by cancer cell (A. Holgado et al. 2012). Another study described doxorubicin-loaded multiwalled carbon nanotube-hyaluronic acid conjugate that targets A549 human lung adenocarcinoma cells. The conjugated system showed higher apoptotic activity and cytotoxicity than free drug in both in vitro and in vivo models (Datir et al. 2012). Moreover, chitosan-modified single-walled carbon nanotubes conjugated with folic acid were reported to selectively deliver doxorubicin to human liver cells SMMC-7721 and kill the hepatocellular carcinoma cell line (Ji et al. 2012).

16.3.6 Inorganic Nanoparticles

Inorganic nanoparticles include metallic nanoparticles such as superparamagnetic iron oxide nanoparticles (SPIONs), gold nanoparticles, silver nanoparticles and silica nanoparticles. SPIONs such as magnetite (Fe_3O_4), maghemite (γ-Fe_2O_3) and hematite (α- Fe_2O_3) are widely explored in nanomedicine because of their low toxicity and good biocompatibility. In addition to their nanostructural characteristics, the magnetic properties exhibited by SPIONs make these materials uniquely suitable for theranostic applications. SPIONs have been used in magnetic resonance imaging (MRI), magnetic separation of biomolecules, targeted drug delivery and hyperthermia cancer therapy (Gupta et al. 2007; Hedayatnasab et al. 2017; Ansari et al. 2017; Patil-Sen and Chhabria 2018). Moreover, core-shell-type nanoparticles where SPIONs are coated with biocompatible materials such as lipids, silica and polymers have been shown to provide multifunctionality and enhance the efficacy of SPIONs for drug delivery and other bio-applications (Majid et al. 2017). Gold nanoparticles have been used as cancer sensors, in drug delivery and imaging (Shipp 2006; Thaxton et al. 2009; Kang et al. 2011). Silica nanoparticles have been used for targeted drug delivery (Hilliard et al. 2002; Zhu et al. 2009).

Inorganic nanoparticles are used in theranostic applications, mainly in imaging and drug delivery. One of the major problems associated with some of these nanoparticles is their cytotoxicity. However, some of the inorganic nanoparticles are in clinical trials and some SPION formulations are FDA approved (Maier-Hauff et al. 2011; Wang 2011).

16.3.7 Cell-Membrane-Coated Nanoparticles

Cell-membrane-coated nanoparticles are biomimetic nanoparticles prepared using a nanoparticle core and membrane(s) derived from natural cells. Membranes can be isolated from cells such as erythrocytes, white blood cells, platelets, mesenchymal stem cells, cancer cells and bacterial cells and can be used to coat different types of nanoparticle core, e.g. polymeric nanoparticles and iron oxide nanoparticles (Hu et al. 2011; Parodi et al. 2012; Antonelli et al. 2011; Wei et al. 2016; Gao et al. 2015; Fang et al. 2014; Lai et al. 2015). Owing to their cell surface mimetic features and engineered nanomaterial characteristics, these materials have gained a lot of attention recently. They have shown strong potential in diagnostic and therapeutic applications including those in immune modulation, vaccination, detoxification and drug delivery.

Zhang and co-workers were the first group to develop the technique of coating nanoparticles with erythrocyte membranes in 2011 (Hu et al. 2011). These particles have shown to increase the circulation time and reduced reticuloendothelial system uptake. White blood cell membrane-coated nanoparticles have been reported to show higher efficacy in the transportation of anticancer drug. Cancer cell membrane-coated nanoparticles have been used for tumour targeting, whereas bacterial cell membrane-coated nanoparticles find applications in vaccination against antimicrobial-resistant bacteria (Gao et al. 2015; Fang et al. 2014).

Although a lot of progress has been made in this field, the research in this area is still in its early stages. There are several challenges that need to be overcome before these particles can make a transition from the bench-to-bed stage. Some of the problems associated with these kinds of nanoparticles are complexity of the fabrication process, short-time stability and heterogeneity in the efficacy. However, considering the promising research so far, these nanostructures hold potential as theranostics (Narain et al. 2017).

16.4 Targeting Approaches Using Nanoparticles

Nanoparticles can be employed as carriers of anticancer drugs to tumour site by active cellular targeting or passive tissue targeting (Fig. 16.3).

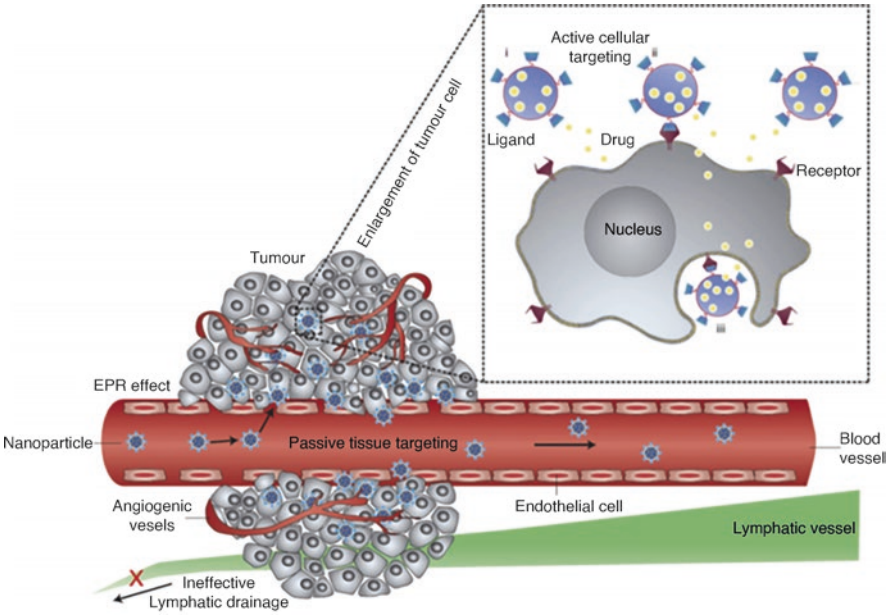

Fig. 16.3 Nanoparticles as promising carriers of anticancer drugs to tumour site by passive tissue targeting and active cellular targeting. (Reproduced with permission from Perez-Herrero and Fernandez-Medarde 2015)

16.4.1 Active Targeting

Active targeting is a strategy to carry the drug to a malignant tumour by attaching specific ligands to the nanoparticles. These ligands can recognize receptors or antigens that are overexpressed by tumour cells (Figs. 16.3 and 16.4).

Active targeting formulations are accumulated in tumour tissues by enhanced permeation and retention (EPR) effect and then internalized by receptor-mediated endocytosis (Bi et al. 2016). Actively targeting nanomedicines that use ligands can enhance endocytosis using receptors such as folate, galactosamine, EGF and transferrin. As the ligand-conjugated nanoparticles are specific to a tumour, they increase the cytotoxicity of the anticancer drugs to the tumour cells but prevent harmful side effects to the rest of the cells by minimizing their exposure to the drugs. Besides allowing active targeting, the functionalization of the nanoparticle surface also enhances the therapeutic efficiency of cytotoxic drugs and helps to overcome multidrug resistance (MDR) (Ganoth et al. 2015).

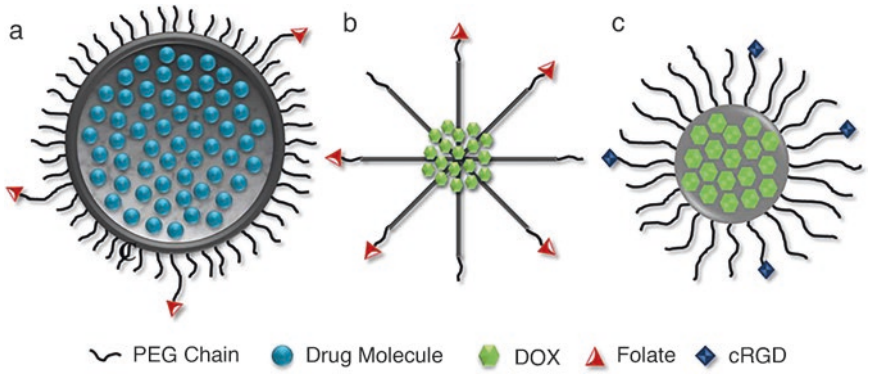

Fig. 16.4 Targeted particles: (**a**) example of a folate receptor-targeted particle. Liposome functionalized with PEG tethers to impart STEALTH characteristics and folate for tumour targeting (**b**) folate-conjugated PLGA-PGA polymeric micelle loaded with encapsulated doxorubicin and (**c**) c arginine–glycine–aspartic acid (RGD) functionalized poly(caprolactone) (PCL)–PEG polymeric micelle containing encapsulated doxorubicin. (Reproduced with permission from Steichen et al. 2013))

16.4.1.1 Albumin-Based Targeting

Apart from accumulating at the tumour site due to the EPR effect, albumin-bound nanoparticles can bind to the glycoprotein 60 (gp60) receptor that aids endothelial transcytosis. Moreover, tumour cells over-express the albumin-binding protein BM-40 (SPARC, osteonectin). Post transcytosis, once the albumin-bound nanoparticles are in the tumour interstitium, binding to SPARC mediates their endocytosis into the tumour cells (Fig. 16.5) (Larsen et al. 2016; Frei 2011; Nitta and Numata 2013).

The albumin nanoparticle, Abraxane (nab-docetaxel), is available in the market, and four others are currently in clinical trials (ABI-008 to 011) (Cortes and Saura 2010). At present, there is an ongoing preclinical study of a new taxane nab of 70 nm including docetaxel (ABI-013). When this formulation was tested in vivo on mouse models of human breast, colon and lung cancer, it exhibited superior activity, better stability and greater antitumour activity than solvent-based docetaxel (L. Mason 2010). Furthermore, studies conducted in monkeys and rats showed ABI-013 to be an effective cytotoxic agent without cardiovascular or central nervous system effects.

16.4.1.2 Hyaluronic-Acid-Based Targeting

Hyaluronic acid (HA) is a polysaccharide present in the extracellular matrix which is necessary for growth, proliferation and adhesion of cells. HA binds to specific cancer cells that overexpress the glycoprotein CD44 receptor. Furthermore, it is biocompatible and biodegradable. Self-assembled amphiphilic hyaluronic acid (HA) nanoparticles, designed by Choi et al., actively target the glycoprotein CD44 which is overexpressed by SCC7 cancer cells (Choi et al. 2012). In vitro studies using fluorescent labelling showed efficient intracellular uptake of the nanoparticles, and fluorescence imaging system indicated in vivo accumulation of the nanoparticles into the tumour after two days in the bloodstream, in tumour-bearing

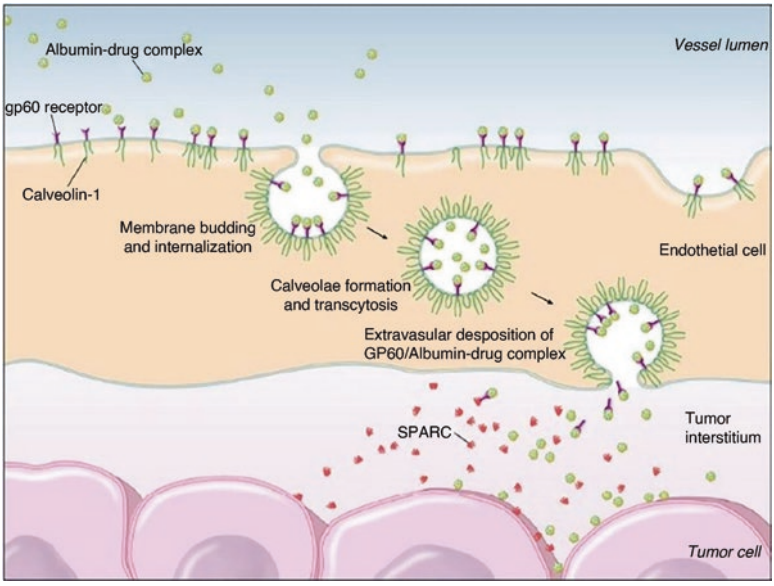

Fig. 16.5 Uptake of albumin-paclitaxel nanoparticles is presumably mediated by the gp60 transcytosis pathway and subsequent binding to SPARC (Secreted Protein, Acidic and Richin Cysteine) in the tumour extracellular matrix. (Reproduced with permission from Nitta and Numata 2013)

mice (Choi et al. 2010). Polyethylene glycosylation of these nanoparticles can improve active and passive tumour targeting in tumour-bearing mice, reduce the liver uptake and increase the circulation time as well as the accumulation of the nanoparticles (Choi et al. 2011a, b).

Cho et al. demonstrated that self-assembled nanoparticles of HA–ceramide conjugate and Pluronic 85 were non-toxic when tested for intravenous delivery of docetaxel (Cho et al. 2011). Due to the small size of the particles (<150 nm) and the external location of the HA, it was possible to deliver docetaxel by passive as well as active targeting. Pluronic 85 increased the solubility of the drug, stabilized it and helped to overcome the multidrug resistance. Increased cell uptake by receptor-mediated endocytosis in CD44-overexpressing MCF-7 cells and evasion of multidrug resistance in MCF-7 and ADR cell lines were observed in in vitro studies. In vivo studies in tumour-bearing mice that overexpress the CD44 receptor using near-infrared fluorescence imaging showed the tumour targeting ability of this formulation.

Two recent studies involved model systems of HA-conjugated nanoparticles loaded with DOX that were designed to overcome MDR in CD44-overexpressing human cancer. In one of these studies, in vitro Q-graphene-based nanocarriers were internalized by HA-mediated endocytosis and increased the cytotoxic effect in MDR lung tumour cell lines (Luo et al. 2016). In the other study, HA-L-lysine methyl ester lipoic acid-conjugated nanoparticles exhibited very high in vivo uptake into human CD-44+ breast cancer xenografts (Zhong et al. 2015).

Combination therapy using the simultaneous delivery of antitumour drugs and chemosensitizers can help to overcome many MDR mechanisms at once. HA-coated nanoparticles carrying mitoxantrone (an anthracycline topoisomerase II inhibitor) and two efflux transporter inhibitors – quercetin (inhibiting P-gp) and hesperetin (inhibiting BCRP) – exhibited in vivo cytotoxicity and bio-distribution that proved their efficacy. Thus, a smaller dosage of drugs was required and side effects were minimized in drug-sensitive and MDR human breast and ovarian cancer cells, both in vitro and in vivo (Zafar et al. 2014).

16.4.1.3 Biotin-Based Targeting

Cancer cells proliferate rapidly and thus require a larger amount of vitamin H or biotin than normal cells. Biotin is an essential cofactor for the enzyme methionine synthase. This enzyme regulates one of two pathways for the production of reduced folates. Breast, lung, colon, renal and ovarian tumour cells overexpress the biotin receptor, presenting a possible way to overcome MDR (Russell-Jones et al. 2004). For this reason, tumours exhibit a higher surface concentration of receptors for biotin than normal tissues. Therefore, it is possible to use biotin as a targeting ligand (Na et al. 2003). Taheri et al. developed human serum albumin (HSA) nanoparticles with biotin ligands for the delivery of methotrexate (Taheri et al. 2011a, b). During in vitro studies, these nanoparticles displayed higher cytotoxicity than non-functionalized particles and free methotrexate. In vivo studies in mice with 4 T1 breast carcinoma that overexpresses the biotin receptor revealed that these nanoparticles increased the effectiveness of the drug and reduced its toxic effects compared to non-functionalized nanoparticles or the free drug.

Patil and co-workers used biotin-functionalized PLGA nanoparticles loaded with paclitaxel and tariquidar (an inhibitor of P-glycoprotein) for drug delivery to drug-resistant tumours. These nanoparticles exhibited enhanced cytotoxicity in vitro and increased inhibition of tumour growth in drug-resistant mouse model (Patil et al. 2009a). Self-assembled amphiphilic poly-(curcumin-dithiodipropionic acid)-b-PEG-biotin copolymer nanoparticles loaded with DOX showed both passive targeting and biotin receptor-mediated active targeting of tumours (Guo et al. 2016).

16.4.1.4 Folate-Based Targeting

Folate or folic acid (vitamin B9) is used as a targeting ligand due to its ability to recognize and bind to cell surface folate receptors (Fig. 16.4). These are primarily overexpressed in epithelial cancers (of the mammary gland, ovary, lung, nose, throat, colon, prostate and brain), hematologic cancers (chronic and acute myelogenous leukaemia) and sarcomas (uterine sarcoma and osteosarcoma) (Hilgenbrink and Low 2005; Sudimack and Lee 2000). When the folate ligand binds to the folate receptor, the conjugated nanoparticle is internalized by the tumour cells. The particle then releases the encapsulated cytotoxic drug into the cytoplasm of the tumour cell (Haley and Frenkel 2008).

Patil et al. functionalized the surface of PLA–PEG nanoparticles loaded with the drug paclitaxel with two kinds of ligands, biotin and folic acid, in a single step. In

vivo testing in a mouse MCF7 tumour xenograft model showed increased accumulation of the nanoparticles in the tumour and high efficacy (Patil et al. 2009b). Nukolova and co-workers developed folate-targeting nanogels using di-block copolymer poly(ethylene oxide)-b-poly(methacrylic acid) (PEO-b-PMA) (Nukolova et al. 2011). During in vitro studies, these showed increased cell uptake in human ovarian carcinomas cell lines that overexpress folate receptors. In vivo studies of the formulation in mouse models with ovarian cancer showed higher antitumour activity and decreased renal toxicity of cisplatin.

Recently, several studies were conducted using folate-conjugated nanoparticles for targeted delivery of doxorubicin (DOX) to tumour site. In one such study, when drug-sensitive human breast cancer MCF-7 cells and their MDR Pgp-overexpressing Dox16-resistant subline were tested, folate-conjugated DOX-loaded nanoparticles displayed >90% loading efficiency, enhanced in vitro cell uptake of DOX and improved antitumour activity with minimum toxicity in vivo compared to free DOX (Lu et al. 2014). Folic acid-conjugated liposomes co-loaded with DOX and C6 ceramide (a pro-apoptotic compound) induced apoptosis in several drug-sensitive and drug-resistant carcinoma cells, thus inhibiting their proliferation (Sriraman et al. 2016).

16.4.1.5 Transferrin-Based Targeting

Transferrin is a membrane glycoprotein that facilitates iron transport to rapidly growing cells. There is a high demand for iron in cancerous tissue, due to which there is overexpression of transferrin receptors (TfRs) on the tumour cell surface (Widera et al. 2003). Transferrin binding to TfR exhibited 10- to 100-fold higher binding efficiency in cancer cells than in normal cells (Guo et al. 2015). Therefore, transferrin-conjugated nanoparticles can be used to deliver drugs to malignant cells (Fig. 16.6). They bind to TfRs, leading to their endocytosis into cancer cells, and then release the anticancer drug into tumour cells (Sahoo et al. 2004).

Hong and co-workers developed stealth TfR-targeting nanoparticles (Tf-PEG-NP) containing a poly(ethylene) glycol–hydroxycamptothecin conjugate (PEG–HCPT conjugate) (Hong et al. 2010). These nanoparticles exhibited a sustained release profile in vitro, as well as better behaviour compared to PEG–HCPT conjugates in S180 solid tumours induced in mice, showing longer circulation times in blood, greater tumour accumulation and enhanced antitumour activity.

Transferrin receptors are overexpressed on the surface of endothelial cells present in the blood–brain barrier (BBB) and can thus be targeted for delivering anticancer drugs to the brain (Visser et al. 2004). Jain et al. developed PLGA–PEG nanoparticles functionalized with transferrin to deliver temozolomide to the brain, displaying higher cytotoxicity in vitro compared to the free drug. In vivo studies in rat brain tissue using fluorescence imaging with confocal laser scanning microscopy showed increased cellular uptake of these nanoparticles and their localization in the brain (Jain et al. 2011).

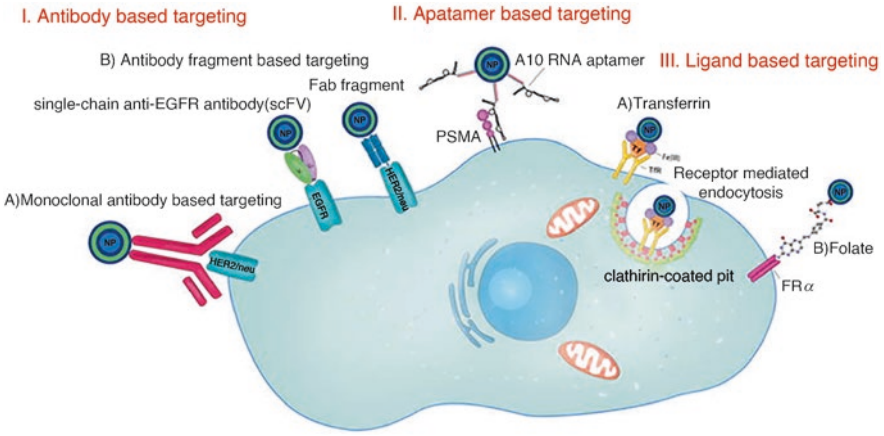

Fig. 16.6 A schematic illustration showing methods used for active targeting of nanoparticles. (I) Antibody-based targeting, which involves the use of *A* monoclonal antibodies such as single-chain anti-epidermal growth factor receptor (EGFR) antibody directed toward EGFR. (II) Aptamer-based targeting such as the A10 RNA aptamer directed towards prostate-specific membrane antigen (PSMA) on the surface of the target cells. (III) Ligand-based targeting such as (A) transferrin-based targeting of nanoparticles towards transferrin receptors. (Reproduced with permission from Bazak et al. 2015)

16.4.1.6 Aptamer-Based Targeting

Aptamers are short nucleic-acid-based ligands which are used for active targeting of tumour cells (Guo et al. 2011). Dhar et al. used A10 aptamer-conjugated PLGA–PEG nanoparticles of about 150 nm in size to deliver cisplatin to prostate cancer cells by targeting the prostate-specific membrane antigen (PSMA), which is overexpressed in prostate tumours (Dhar et al. 2008) (Fig. 16.6). The formulation was 80 times more cytotoxic to cancer cells than the free drug, and studies in rat and mouse models indicated a decrease in the size of tumours at a significantly lower drug dose (Farokhzad et al. 2004a). Guo et al. designed PEG–PLGA nanoparticles conjugated to AS1411, a DNA aptamer that recognizes and binds to nucleolin (a protein that is overexpressed in the plasma membrane of cancer cells) (Guo et al. 2011). This formulation was used to deliver the paclitaxel drug to C6 glioma cells, thus enhancing the anti-glioma efficiency of the commercial form of the drug. As a result, longer circulation time and enhanced cytotoxicity of the drug were attained.

Aptamer-tethered DNA nanodevices (aptNDs) are capable of specifically recognizing cancer cells and self-assemble in situ on the surface of target living cells. The ligands present in these structures, aptamer sgc8, bind to human protein tyrosine kinase 7, which is overexpressed on CCRF-CEM (human T-cell acute lymphocytic leukaemia) cell membrane. Cancer cells can be detected by attaching fluorophores on each nanodevice or using electrochemical luminescence methods. The sensitivity and selectivity of this detection can be enhanced by using aptamer-DNA concatamer-quantum dot probes for signal amplification and can detect around 50 cells

per millilitre (Wu et al. 2013). Bamrungsap et al. designed aptamer-conjugated magnetic nanoparticles (ACMNPs), which use the ability of sgc8 aptamer to specifically bind target cancer cells (Bamrungsap et al. 2012). The large surface areas of magnetic nanoparticles (MNPs) allow multiple aptamer-binding events. As most biological samples have negligible magnetism, MNPs can be used for sensitive detection of cancer cells. The ACMNPs are highly sensitive and can detect 10 cancer cells in a 250 ml sample (Jie et al. 2011).

16.4.1.7 Monoclonal-Antibody-Based Targeting

Monoclonal antibodies (mAb), which are specific to antigens or receptors overexpressed in tumours, can be used to target cancer cells by conjugating them to the surface of nanoparticles (immuno-nanoparticles) (Fig. 16.6).

One common target of mAb used in clinical applications is the EGFR, as it is overexpressed in a broad spectrum of tumours (bladder, breast, ovarian, prostate, non-small cell lung, head and neck cancers and glioblastoma). Polymeric poly(lactide-co-glycolide) nanoparticles functionalized with an EGFR antibody are loaded with rapamycin and used to target the extracellular ligand-binding domain of EGFR present on tumour cells (Acharya et al. 2009). This formulation facilitated inhibition of cell proliferation more than both the nanoparticles without the targeting ligand and free rapamycin. Furthermore, confocal microscopy and fluorescence spectroscopy revealed higher uptake in malignant MCF-7 breast cancer cells, and flow cytometry studies revealed that the formulation induced cell cycle arrest and apoptosis.

HER2, which is overexpressed in breast and ovarian cancers, belongs to the EGFR family and is associated with angiogenic processes (Slamon et al. 1989). Polymeric nanoparticles functionalized with anti-HER2 mAbs can bind to specific antigens on the tumour cells and are then internalized by receptor-mediated endocytosis (Cho et al. 2008). Cirstoiu-Hapca and co-workers developed poly(DL-lactic acid) nanoparticles covalently conjugated to anti-HER2 mAbs (trastuzumab) and loaded with paclitaxel and used these immuno-nanoparticles to target ovarian tumour cells that overexpress HER2 receptors (Cirstoiu-Hapca et al. 2009). In vitro studies revealed that these immuno-nanoparticles had improved cytotoxicity at low drug concentrations compared to nanoparticles without the anti-HER2 mAbs, nanoparticles with irrelevant mAbs and free paclitaxel. Bioluminescence imaging of this formulation in a xenograft ovarian cancer mouse model showed that the immuno-nanoparticles had higher antitumour activity and longer mouse survival rate than free paclitaxel (Cirstoiu-Hapca et al. 2010). Trastuzumab-conjugated human serum albumin (HSA) nanoparticles were also used to deliver the drug methotrexate to tumours and were more cytotoxic and had greater specific uptake than the free drug and non-conjugated nanoparticles (Taheri et al. 2012).

Besides being used for small molecular weight drug delivery, immuno-nanoparticles can also be used for macromolecule (genes and proteins) delivery. Chen et al. developed PE38KDEL-loaded PLGA immuno-nanoparticles conjugated with the Fab' (fragments antigen binding) of a humanized anti-HER2 monoclonal antibody (Chen et al. 2008). In vitro, the presence of the Fab ligand enhanced the

cytotoxicity of formulation against breast cancer cell lines that overexpress HER2 receptors. In vivo, the formulation exhibited higher inhibition of the tumour growth than the control immunotoxin (anti-HER2 Fab conjugated to the protein toxin PE38KDEL) in a tumour xenograft mouse model overexpressing the HER2 receptor.

16.4.2 Passive Targeting

Many nano-biomaterial platforms such as liposomes, polymers and micelles use passive targeting to increase the circulation time of conjugated or encapsulated anti-cancer drugs (Zhao et al. 2005). These nanoplatforms accumulate in tumours over time by the EPR (Fang et al. 2011) (Fig. 16.3). The EPR effect is an angiogenic process causing the occurrence of highly permeable blood vessels in tumours due to the presence of trans-vascular endothelium gaps (100 nm and 780 nm) (Wicki et al. 2015). This causes an abnormal level of lymphatic drainage, due to which nanoparticles can accumulate in tumours and release cytotoxic drugs near tumour cells (Wang and Thanou 2010).

The first formulation to be approved for cancer treatment was Doxil®/Caelyx® (Brown and Patel 2015). Another example, Abraxane®, is an albumin-bound paclitaxel (PTX) formulated as injectable nano-spheres (~130 nm) (Stinchcombe 2007). Both above examples were unable to overcome drug resistance. Other clinically approved passively targeted nanomedicines include Myocet (nonpegylated liposomal doxorubicin), Oncaspar (pegylated L-asparaginase) and Abraxane (albumin-based paclitaxel) (Gelderblom et al. 2001; Brown and Patel 2015). Similarly, several single compounds were delivered using nanoplatforms that were designed based on the EPR effect and used non-specific, non-receptor-mediated endocytosis (Wicki et al. 2015).

Many passive targeting nanomedicines are presently in clinical trials, while others are in early- and late-stage preclinical development (Peer et al. 2007). Recently, phase I clinical trials as well as a pharmacokinetics study of PEGylated liposome-encapsulated irinotecan (a topoisomerase I inhibitor) showed high and prolonged exposure to both irinotecan and its bioactive metabolite (SN-38) in 60 patients with different types of advanced solid tumours (Infante et al. 2012). Similarly, a phase I trial in 41 previously treated patients with advanced malignancies using dose escalation of SN-38-conjugated PEG exhibited prolonged exposure to SN-38 and decreased harmful side effects compared to irinotecan (Patnaik et al. 2013). There have been publications reported in the literature describing clinical studies of liposome-entrapped PTX. The first clinical trial involved 58 metastatic gastric cancer patients which was carried out to compare the safety and efficiency of liposomal PTX and free PTX (Xu et al. 2013). Despite similar response rate in both cases, liposomal PTX had much lower harmful side effects (nausea, vomiting and hypersensitive reactions). The second study used pharmacokinetics to compare

liposomal-PTX and PTX solubilized in polyethoxylated castor oil. Both formulations were discovered to be bioequivalent (Slingerland et al. 2013). The third study was a phase II trial of 143 patients with advanced triple-negative breast cancer (do not express oestrogen receptor, progesterone receptor and HER-2) (Awada et al. 2014; Schmitt-Sody et al. 2003). It utilized a cationic liposome with a lipid-embedded PTX (EndoTAG™-1), which targeted negatively charged, active endothelial cells of tumours using the EPR effect. It was observed that combination treatment of EndoTAG™-1 with standard PTX had efficient antitumour activity and exhibited manageable undesirable effects.

PLGA curcumin nanoparticles were shown to block the multidrug efflux activity of Pgp on CAL27 cisplatin-resistant human oral cancer cells (Chang et al. 2013). D-α-Tocopheryl polyethylene glycol succinate (TPGS) is a vitamin E and PEG 1000 derivative which can inhibit Pgp-dependent drug efflux by suppressing the production of ATP in mitochondria (Wang et al. 2012b). Micelles of pH-responsive di-block copolymer and TPGS loaded with DOX were shown to effectively inhibit tumour growth in an orthotopic MDR breast cancer mouse model (Yu et al. 2015). Moreover, this formulation also reduced bio-distribution of cardiotoxic DOX to the heart. Mice treated with free DOX showed severe weight loss compared to those given DOX-loaded micelles, demonstrating the lower toxicity of the latter. Also, TPGS-incorporating PTX nano-emulsion in drug-resistant human breast cancer xenografts exhibited increased tumour growth inhibition (> 90%) when compared to free PTX (around 60%) (Bu et al. 2014). Next-generation anticancer nanomedicines focus on minimization of the side effects of surfactants in nanoparticle formulations and improved targeting of tumour tissues.

16.5 Conclusions and Future Perspectives

Nanoparticles present promising drug carrier systems for the therapeutic agent and potentially improve the cancer treatments by delivering the drug to the target cancer site. Moreover, nanoparticles offer opportunities to develop delivery systems with improved circulation half-life and pharmacokinetic, and enhanced bioavailability and biodistribution. Thus, a wide spectrum of nanoparticle structures and targeting ligands have been explored over the past several years (Table 16.1). However, a very few of these nanocarriers have so far been FDA approved. Complex manufacturing processes, stability issues and toxicity are some of the major concerns associated with these nanostructures, which hamper their widespread applications in nanomedicine. Nonetheless, these challenges provide new opportunities for researchers to find ways to develop new formulations with superior quality. Thus, the field of nanomedicine will continue to expand to reach its full potential. Furthermore, nanoparticle carriers have the potential to revolutionize the field of oncology and to improve both the cancer treatment and quality of patient's life.

Table 16.1 Recent reports on nanoparticles against cancer

Sr No.	Nanostructured compound	Type of cancer	Properties of nanoparticles	Application	Reference
1	Pheophorbide-a (P@)-gem-human serum albumin (HSA)-NPs	Pancreatic cancer	Monitor drug delivery and inhibit pancreatic cancer with lymphatic metastases	Photodynamic therapy and chemotherapy	Yu et al. (2017a)
2	p53/candesartan/amine-functionalized hydroxyapatite NPs	Breast cancer	Effectively transfer the p53 gene and deliver the loaded candesartan	Anti-angiogenesis therapy	Zhao et al. (2017)
3	Multiwalled carbon nanotubes@ poly(N-vinyl pyrrole)-S-polyethylene glycol-folic acid	Various types of cancers	High drug-loading ratio and pH-sensitive unloading capacity	Chemo-photothermal therapy	Wang et al. (2017)
4	Chlorin e6–polyethylene glycol–gold nanorods	Various types of cancers	Stable at normal physiological condition and better results of combination therapy than the PDT/PTT alone	Combination with photodynamic (PDT)/ photothermal (PTT) therapy	Zhang et al. (2017a)
5	CRRK(RRCG(Fmoc))2 loaded with doxorubicin nano-micelles	Various types of tumours	Beneficial in case of multidrug-resistant tumours	Drug delivery system	Chen et al. (2017)
6	Alpha-cyclodextrin (a-CD)-based gold/DNA nanomachine	Various types of tumours	Tumour-specific targeting and enhanced retention	Photoacoustic imaging and photothermal therapy	Yu et al. (2017b)
7	PAMAM-histidine-PEG-triptorelin nano-constructs	Breast and prostate cancer	Good binding capacity for siRNA with excellent serum stability and negligible toxicity	siRNA delivery, gene silencing and tumour therapy	Tambe et al. (2017)

8	Paclitaxel and borneol co-delivery dendrimer-derivative PEG-PAMAM NPs	Ovarian Cancer	Promising drug delivery for multidrug resistance overcoming	Combination chemotherapy	Zou et al. (2017)
9	Bifunctional tellurium nanodots	Various types of cancers	Good tissue transparence, ultra-small size, ideal photostability, synergistic effect through effective cytoplasmic translocation, preferable tumour accumulation and easy renal excretion, thereby facilitating total tumour ablation	Photo-induced synergistic cancer therapy	Yang et al. (2017)
10	Nanoscale coordination polymers-chlorin e6-doxorubicin-PEG nanostructures	Various types of tumours	Biodegradability, sensitive responses to light and highly efficient tumour retention	Combined chemo-photodynamic therapy	Liu et al. (2017)
11	DNA origami, AuNRs and molecular targeted anticancer drugs into Ca3(PO4)2 nanoparticles coated with phospholipid DOPC.	Various types of cancer	Biodegradable, very good biocompatibility and high drug-loading capacity	Synergistic chemotherapy and induce apoptosis	Zhang et al. (2017b)
12	Tetraiodothyroacetic acid-conjugated PEG–PLGA polymersomes carrying camptothecin	Colon adenocarcinoma	Greater accumulation, increased distribution in tumour tissue cytotoxicity	Targeted drug delivery	Alibolandi et al. (2017)

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Preclinical Non-invasive Imaging in Cancer Research and Drug Discovery: An Overview

17

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Abstract

The increased understanding of complex biological processes in cancer initiation and progression has proportionately increased new anticancer drug development which is presently the top priority research field. The process of drug discovery and development is expensive, tedious, and unpredictable. Several steps are involved in this process, which requires strong multidisciplinary interaction. The duration of the procedure depends upon the validation of each

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step. Current data shows that out of the 10,000 newly screened compounds, only 250 enter preclinical testing and 5 enter clinical phase. However, only one compound gets regulatory approval. The percentage of success in developing a drug is very low, in spite of huge time spent and financial investments. Therefore, it is utmost important to refine every step of the drug development process so as to precisely evaluate the new compounds. The process of drug development consists of various phases like absorption, distribution, metabolism and excretion. All these phases can be precisely studied using several advanced tools and one such tool is non-invasive preclinical imaging. This chapter deals with various preclinical imaging modalities having huge potential in anticancer drug discovery. The imaging modalities discussed in details are positron emission tomography (PET), single photon emission computed tomography (SPECT), computed tomography (CT), optical, ultrasound and photoacoustic (US-PA) and spectral imaging. These modalities are complimentary to each other and need to be applied on the basis of information to be gathered. The common study/imaging protocol used in anticancer drug development has been mentioned with appropriate image data. The modalities such as photoacoustic and spectral imaging have huge potential for clinical applications due to their potential to characterise tissue and underlying pathophysiological differences.

Keywords

Preclinical imaging · Animal model · Positron emission tomography · Single photon emission computed tomography · Ultrasound · Photoacoustic imaging and spectral imaging

The development process of new drug is expensive, tedious, and unpredictable process. Several steps are involved in the development process requiring strong interaction between many departments. The length of the procedure depends upon the validation of each step. Starting from the screening of compound, around 10,000 compounds get discovered, 250 enter the preclinical testing, only 5 enter the clinical phase, and only one gets approved by regulatory bodies. The percentage of success

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is very low even with a huge investment of around $1.7 billion and time of approximately 10 years for complete drug discovery and development process (DiMasi et al. 2003, 2010, 2016). Therefore, it is utmost important to add steps, which reduce the time of drug discovery and validate the compounds at an early stage and help to make decisive call whether to continue with that compound or drop it at earlier stage. This will save time and money and increase the confidence to take molecules further for human clinical trials.

The drug development consists of various phases, and investigators must gather information regarding various processes like absorption, distribution, metabolism and excretion. The compound must be explored for its potential benefits, mechanism of action, exact dosage for treatment and route of administration. The drug must be tested in vitro and in preclinical phase and then clinical phase. The US Food and Drug Administration has modified the guidelines to fasten the drug discovery process. Investigational new drug application (INDA) can be applied after successful preclinical studies. If INDA submission is accepted, then the compound is forwarded to the clinical research phase (I–IV studies) (Katz 2004) (Fig. 17.1).

In view of the huge investments and time consumption, pharma companies are always looking for options or methods that can decrease the time of drug discovery and development. Molecular imaging has provided a huge boost to the drug development process. Preclinical imaging modalities can be helpful in the initial stages of the drug development. It can provide evidence of biodistribution, pharmacokinetics, target confirmation, drug effects, treatment response assessment, identifying responders from non-responders and many more. Therefore, investment in the imaging modality technology can fasten the drug development program and save cost and time.

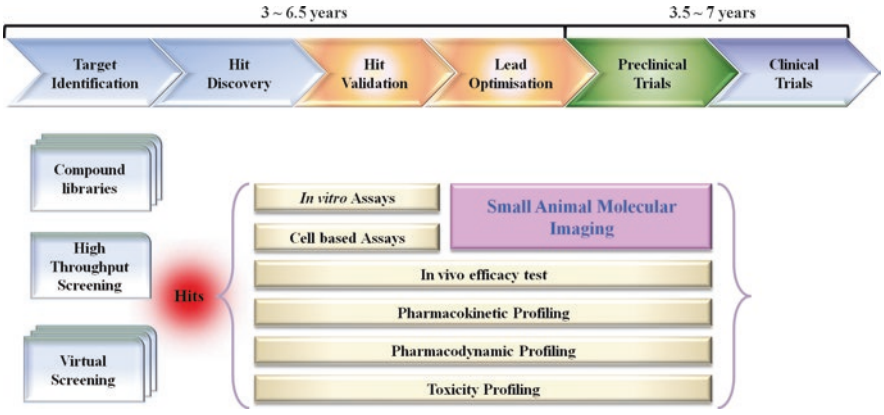

Fig. 17.1 Flowchart showing the route of drug development

In this chapter, we have reviewed the basic principles of imaging modalities and their contribution in various phases of the drug development and discovery. There are two subchapters on advanced imaging modalities, such as photoacoustic imaging and spectral imaging, which are going to be promising modalities in coming years.

17.1 Molecular Imaging

The term 'molecular imaging' defines the capability of a modality to quantify the biological events at a subcellular level in living subjects. Molecular imaging is a multidisciplinary art which requires coordination and integration of many disciplines including biology, chemistry, pharmacology, medical physics, engineering, mathematics and bioinformatics.

It provides an insight into the organs at cellular level and provides detailed information about any physiological changes, cell integrity and characterisation of the disease. The advancement in the imaging technology has provided high spatial and temporal resolution. The imaging allows repetitive, non-invasive and uniform imaging of the same subject at different time points. It increases the statistical power of the study and reduces the number of subjects required for the study. This helps in reducing the number of animals used to do kinetics, distribution and serial tissue distribution, as all these data can be generated from the imaging of small number of animals. Non-invasive imaging has played a significant role in the process of the drug testing. Imaging has been used to assess left ventricular function, pulmonary artery pressure, myocardial ischaemia and arterial morphology in preclinical models and humans. It provides proof of concept about mechanism of action required for the drug approval (Tarkin et al. 2018).

Molecular imaging provides high-resolution images of organs and lesions to prove diagnosis. The capability of providing quantitative results in preclinical and clinical phase of drug development eases the process of decision-making of whether to continue with the same compound or not. In the preclinical phase, the data provided by imaging deduce distribution and kinetics and monitor the response of the interventions at variable time points in a longitudinal study using the same set of animals, thereby reducing animal mortality. It can provide information about drug dosage and determine the doses of a new pharmaceutical that perturb metabolism, physiology or morphology. The imaging in early phases can provide evidence of biological activity of pharmaceuticals as well as can help to define optimum dose. It gives most useful information which can be handy in determining to-go or no-go decisions for progression of the drug development process to later phases (Frank and Hargreaves 2003). Certain imaging results can be used to support outcomes of phase III studies and subsequently provide post-marketing information. It provides

strong support in assessing the safety profile aspect of the drug under investigation, for example, by providing cardiac or hepatic or renal function measurements. The utility of imaging at each step in the drug development process means that it (alongside numerous other assessment methods) can significantly affect the progress of a drug from the in vivo measurements to regulatory approval. The non-invasive, localised and repeatable nature of imaging means that imaging biomarkers can often provide useful information with relatively small numbers of participants, providing tools for early identification of drug effects. Deployed wisely, imaging can therefore be a cost-effective and powerful component of drug development strategies (Cohen 2003).

There are a number of modalities which are used for non-invasive imaging and have their own advantages and limitations. The radiologic modalities like ultrasound (US), computed tomography (CT) and magnetic resonance imaging (MRI) provide anatomical details of the organ, whereas the functional/molecular imaging modalities like single photon emission computed tomography (SPECT) and positron imaging tomography (PET) provide functional and molecular information. The other imaging modalities like optical, photoacoustic, spectral and infrared imaging and cryofluorescence tomography are gaining popularity too. The most common modalities used for imaging are MRI, PET and SPECT due to their high resolution and sensitivity over CT, US and optical imaging.

17.2 Imaging Modalities

MRI, SPECT and PET play a major role in the drug development. They have different working principles with their own advantages and limitations (Table 17.1). Each system has its own uniqueness and contributes significantly. They play huge role in preclinical testing and provide insight about pharmacokinetics and pharmacodynamics of the drug in the humans in the initial phase of drug trial.

Imaging modalities are primarily divided into anatomical/morphological and functional imaging. Imaging modalities like CT, MRI and US provide anatomical details of the organ/tissue of the subject, whereas optical, PET and SPECT provide molecular/functional details of the particular organ/tissue of the subject. CT and MRI have high resolution and provide structural changes, whereas PET and SPECT offer potential to detect molecular and cellular changes which gives it a potential to detect tumour or disease at an early stage when molecular changes start appearing in the cells or tissue before the structural changes are seen in the tissue, but they offer a poor spatial resolution. Therefore, the current era belongs to the hybrid imaging where strength of structural and functional imaging combines and provides anatomical and functional details in one image with high sensitivity

Table 17.1 Characteristics of molecular imaging modalities

Modality	Advantage	Disadvantage	Application
Ultrasound	Better resolution	Cannot image bones	Preclinical and clinical
	Provides anatomical and physiological data		
	Portable and cheaper technology		
	No radioactivity usage		
CT	Better resolution for anatomy	No physiological data	Preclinical, clinical and in vitro
	Can provide 3D images	Low soft tissue resolution	
		Expensive setup	
MRI	Best soft tissue delineation	Long image acquisition protocols cause inconvenience	Preclinical and clinical
	Capable of providing anatomical and functional data (fMRI) of the image		
	Zero radiation exposure	Expensive setup	
SPECT	Provide functional imaging data	Lower resolution than PET	Preclinical and clinical
	Cheaper option as compared to PET	Involves radioisotope usage and radiation exposure	
PET	Provides molecular information of the cell processes	Requires on-site cyclotron or procurement of radioactivity	Preclinical and clinical
	High sensitivity as compared to PET	Radiation exposure to the patient	
	Adds critical information to the diagnosis, staging and treatment response assessment of cancers	An expensive setup	
Optical imaging	Molecular events can be noted	Application limited to ex vivo studies and to the animals	Preclinical
	Cheaper than other available imaging technologies		
	No radiation involved		
Multimodal imaging ultrasound and photoacoustic imaging (US-PA)	Wide applicability	Increased sensitivity with functional information	Preclinical and initiated clinical studies
	Hybrid modality combining optical and ultrasound technology		
	Provides 2D/3D and whole-body imaging information		
	Relatively inexpensive		
	No radioactivity usage		
MARS spectral imaging	Wide applicability	Less sensitive to contrast agent than PET	Preclinical and initiated clinical studies
	Provides material-based analysis of subject additional to x-ray attenuation information		
	No radioactivity usage		

and spatial resolution. The rise of hybrid modality like PET-CT, SPECT-CT and PET-MR technology has revolutionised the management of the diseases and has the potential to accelerate the drug development process (Mouchess et al. 2006; Chang et al. 2007; Mendoza-Sánchez et al. 2010; Vergez et al. 2010; Walter et al. 2010). Many pharmaceutical companies have now set up or collaborated with imaging facilities. The imaging has been increasingly applied at the various stages of the drugs.

17.3 Ultrasound

The ultrasound uses high-frequency sound waves and their echoes to image the soft organs of the body. The waves transmit with a frequency of 1–5 MHz, which travels through the body, hit the boundary of the tissues and reflected back to the probe which subsequently relayed towards the machine. The image is created based on the echoes received from the organ. This technique is well established for assessment of myocardial function and perfusion and for characterisation of liver lesions (Piscaglia et al. 2006; Pysz and Willmann 2011). The first ultrasound contrast agent named LUMASON has been approved by FDA for liver imaging. The ultrasonography sensitivity and specificity have been improved for the differentiation between malignant and benign hepatic lesions (Barr 2017). Pochon et al. have incorporated a human VEGFR2-targeted lipopeptide into the microbubble shell and assemble a clinical grade molecularly targeted ultrasound contrast agent (BR55). This contrast agent was injected into an orthotopic breast tumour model in rats by ultrasound imaging (Pochon et al. 2010). Psyz et al. have showed that these could be used for quantification and monitoring of tumour angiogenesis during anti-angiogenic treatment of human colon cancer xenografts in mice. As microbubbles carry drugs against tumor expressing angiogenesis marker, like VEGFR2 and $\alpha_v\beta_3$ integrin, US can be used to track the delivery of the microbubbles and monitor the treatment response (Pysz et al. 2010).

Guibal et al. have studied the effect of discontinuation of the anti-angiogenic therapy drug, bevacizumab, in orthotropic model of renal cancer, and the effect of treatment was evaluated by contrast-enhanced US. The treatment effect was quantified using ultrasound imaging (Guibal et al. 2010). The development in the US imaging has led to three-dimensional (3D) US which are more accurate and precise than manual caliper in measuring the size of the tumour. It is helpful in measuring the tumour size in a longitudinal study during the course of therapy. A number of studies have proved that evaluating the treatment response of drug targeting microvessel density using contrast-enhanced US is more sensitive than evaluation by histological methods. The major advantages of US are that it is inexpensive, is free of radiation, and poses a minimum risk to the subject under study. The major limitation is that it is unsuitable for imaging bone and brain tumours (Xuan et al. 2007; Deshpande et al. 2010; Hoyt et al. 2010).

17.4 Computed Tomography

CT is the most common modality to visualise any anatomical abnormalities in the body. It is based on transmission of a series of X-rays through the body and generating 3D image of the organ or body region. The diagnosis is based on the difference in the tissue density in the normal and abnormal area. A progressive development has been made in the preclinical CT scanner, offering high-resolution, detailed anatomy of the small animals. It has been used for detection of metastasis, pheochromocytoma tumours in the livers of the mouse models, designing treatment strategies for treating tumours (Ohta et al. 2006; Morgan et al. 2008; Kang et al. 2010). Similarly, it has been used to measure bone and tumour volumes in the evaluation of new treatment response and follow-up tumorigenesis using the micro-CT colonography. Therefore, the treatment response of these cancers can be monitored using CT scans (Durkee et al. 2008; Fushiki et al. 2009). The major limitation of CT scan is its resolution, which often falls short and leads to poor scan quality and reader ability. The poor contrast between soft tissues makes it difficult to read out scan results (Schambach et al. 2010; Prajapati and Keller 2011). Therefore, the use of MRI has been increased in preclinical studies as it has no radiation and has high resolution in comparison to CT mainly for soft tissue imaging. The drug response evaluation of brain orthotopic tumours in mouse can also be studied very well using micro-CT imaging (Prabhu et al. 2017) (Fig. 17.2).

In addition to the role of CT scan in diagnosing cancer, it is also used in the diagnosis of non-malignant or bone diseases, such as osteoporosis, and osteoarthritis, and in dental implant evaluation (Das et al. 2018; Thummuri et al. 2017) (Fig. 17.3).

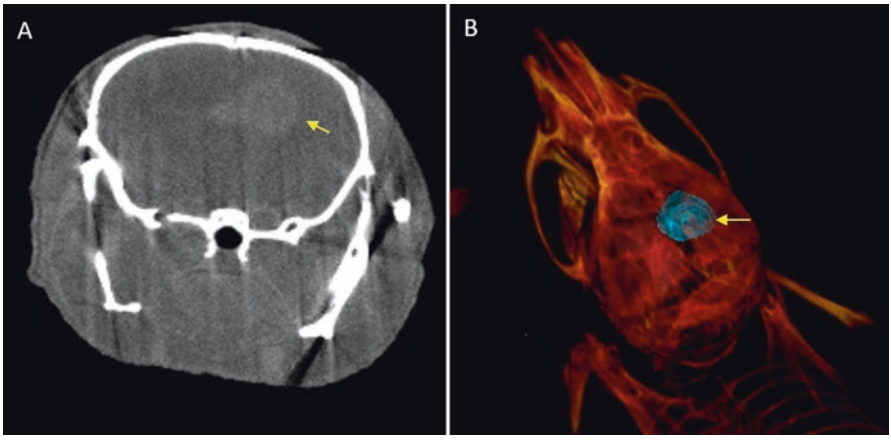

Fig. 17.2 CT scan images in 2D (**a**) and 3D (**b**) view of a glioblastoma brain tumour in mice model

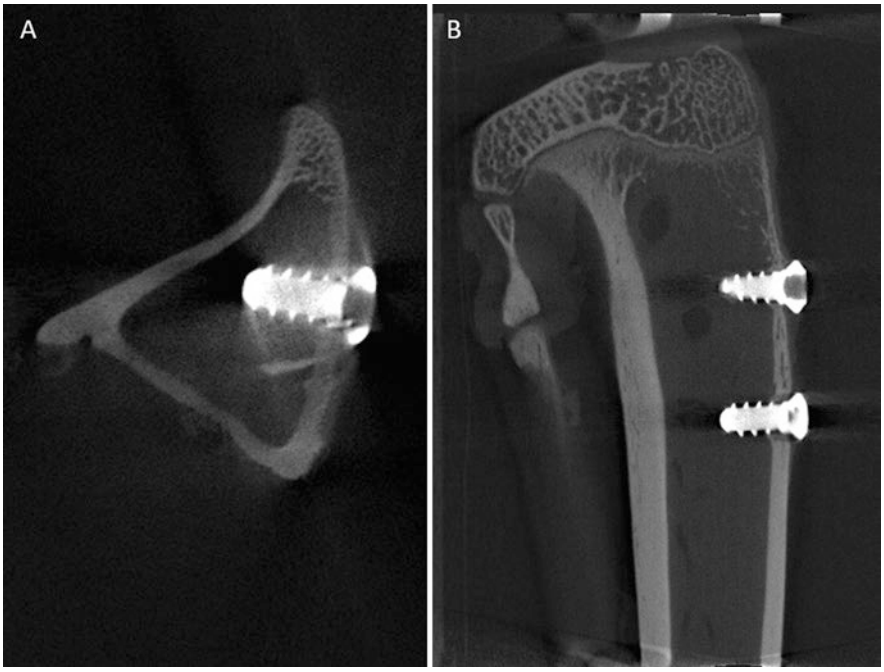

Fig. 17.3 CT scan images of tibia of rabbit in axial (**a**) and sagittal (**b**) view showing dental implants placed at proximal end of tibia

17.5 Magnetic Resonance Imaging

MRI is mainly considered for soft tissues like the brain due to its high resolution. The working principle of the MRI is based on exploiting the magnetic properties of the certain atomic nuclei, hydrogen (^{1}H), under the influence of the external magnetic field. The body of any living organism is made up of water molecules; the hydrogen atom of water under the influence of external magnetic field gets aligned in one direction and when it returns to equilibrium, it emits a radio signal. These signals are detected using coils and detector which form an image on the screen. The major advantage of MRI is precise delineation of soft tissues and does not involve any radiation. It works in the normal radio-frequency range (40–130 MHz) and has no adverse health. Though many factors influence imaging, MRI can be sensitized to the passive diffusion of the water within and between the cells and macroscopic structures of tissues can influence the observed image signal. The changes in MRI signal can be quantified and alterations can be tracked within a tissue, such as tumour tissue. Any drug that acts on tumour cellularity, reduces cell swelling, shrinkage or cell death that often precedes changes in tumour volume, their treatment response can be monitored by MRI (Fomchenko and Holland 2006; Hsueh et al. 2006).

The major contribution of the MRI in drug development is the evaluation of the drug response in animal disease model at an early phase of clinical trial. MRI can translate preclinical data to clinical levels. Therefore, it can be applied to most of the phases of the drug development (Lyons 2005). The role of the MRI starts from the preclinical level and gets along up to clinical levels. The therapeutic drug under trial can be tested in phenotype genetically modified and conventional animal models at an early stage of drug development. The preclinical results can be translated, and MRI also contributes in monitoring the post-therapy response (Grange et al. 2010).

MRI can assess the treatment response of the drug (under investigation) on the anatomy and physiology of the diseased organs in experimental animals or patients, though it has its own limitations of being expensive and provides limited spatial and/or temporal resolution of images and image series of small animals. MRI has been in competition to the other cheaper alternative imaging systems, but superior image quality and greater soft tissue contrast gives it an edge above other imaging modalities. MRI plays a vital role in drug development and as it provide imaging evidence which leads to translate a preclinical study into clinics. The non-invasive imaging allows to do time course studies in the same set of animals and evaluation of the treatment response in the longitudinal studies (Schröder et al. 2006;, Hasegawa et al. 2010).

Multiple applications of MRI in a study give it an upper hand over other modalities. It gives confidence in translating the study to clinical phase and the capability of assessing functional and physiological parameters added to the data. The sequential studies allow time course studies thereby reducing the number of animals used per experiment (Schröder et al. 2006; Hasegawa et al. 2010). Dynamic contrast-enhanced (DCE) MRI has been used frequently for the assessment of the anti-angiogenic and vascular disrupting compounds at the early phase of clinical trials. The improvement in parameters like data acquisition, image processing and analysis ensures that the vital information can be harnessed from the MRI data in the early process of drug development which closely correlates with histopathology outcome (O'Connor et al. 2007). DCE-MRI has proved its utility in monitoring angiogenesis as tumour growth is assigned to enhance vascular density, vessel size and permeability. MRI is non-invasive, which provides an advantage over other techniques. It can also be used to monitor the treatment response in patients undergoing surgical resection of their tumours. DCE-MRI has been established for the quantification of angiogenesis and, therefore, it can be used for monitoring the treatment response in preclinical and clinical research. This technology can assess the anatomy and physiology of the vessel formation. The treatment response was well correlated with histological findings (Tuncbilek et al. 2009).

Due to its non-invasiveness, MRI can be used in longitudinal studies. The pre-treatment baseline scan of the animal can act as control, and then during post-treatment, the same animal can be used for the assessment of the treatment response. It reduces variation in data and increases the confidence and statistical power of the study. Hausler et al. used dogs as a model to characterize the effects of 5α reductase inhibitor on prostate hyperplasia (Häusler et al. 1996). These studies proved how non-invasive imaging can reduce the number of animals to be used and increase the statistical power of the study.

MRI can evaluate the underlying pathophysiology events and therefore can assess the treatment effect on the target organ. One of the examples is of cerebral ischaemia which is induced by the middle cerebral artery occlusion (MCAO). Three-dimensional time-of-flight scanner in the MR angiography can visualise the cerebral vasculature of the rat without contrast agent. MR has played a decisive role in the diagnosis and treatment response monitoring of multiple sclerosis (MS). The soft tissue contrast provided by MRI allows high-sensitivity detection of MS lesions, which are poorly visualised by other non-invasive imaging methods. The lesions typically observed on MRI reflect the localised inflammation often associated with MS lesions and the associated destruction of tissue components. MRI measures lesion number and overall lesion load and has proved useful in monitoring the disease-modifying effects of new pharmaceuticals. Such imaging biomarkers can serve in proof-of-concept studies and dose-finding studies. Other MRI-derived markers, such as brain atrophy measurements, may also have a role to play in some circumstances for trials in MS (Barkhof et al. 2012; Miller 2002).

Functional MRI (fMRI) measures brain activity by detecting changes associated with blood flow predominantly provided by blood oxygenation level-dependent (BOLD) or arterial spin labelling (ASL) perfusion MRI sequences. fMRI has the potential to evaluate the impact of drugs on tissues and differentiate between treatment responders and non-responders. Many other approaches lack predictive validity to complex human neuropsychiatric disorders. In early phase clinical studies, fMRI methods provide a means to detect a functional CNS effect of pharmacological treatment in the brain regions. It can provide a link between fMRI response and the molecular target. Dose response and exposure response relationship using fMRI make a way to guide dose selection for later phases. fMRI has the potential to qualify as a drug development tool in near future.

17.6 Single Photon Emission Computed Tomography

Nuclear medicine imaging is based on the type of radionuclide and energy it emits, for example, energy emitted in a range of 100–300 KeV is suitable for SPECT imaging. Therefore, technetium-99m [^{99m}Tc] is the choice of radionuclide for SPECT imaging as it emits energy in the range of 142 KeV. The gamma rays emitted by radionuclide strike the scintillation crystals which are coupled to the array of photomultiplier tubes. These rays are converted into electric signals and converted into multiple two dimensions which can generate 3D image to delineate the lesions accurately (O'Connor and Kemp 2006; Madsen 2007; DiFilippo 2008). It uses the γ-rays emitting radionuclide like technetium-99m [^{99m}Tc] which emits radiation in the range of 30–300 keV. [^{99m}Tc] (half-life 6 h) is the work horse of nuclear medicine for gamma imaging. Its labelling chemistry is easy which makes it convenient to label with drug under development and study its PD or PK. A pico- or nanogram of drug is required to study PD or PK of the drug. The drug will be labelled with [^{99m}Tc] and injected into animals or volunteers, and then SPECT imaging will be done at various time intervals. A half-life of 6 h gives convenience of doing delayed

imaging even at 24 h. In present scenario, many drugs fail due to inappropriate pharmacokinetics and metabolism despite of extensive preclinical studies (Johnson et al. 2001; DiMasi et al. 2003).

The high sensitivity of SPECT imaging provides the pharmacokinetics data of drug using microgram of drug in the radiolabelled form in mouse and human volunteers. The imaging data can provide real-time deposition data, pharmacokinetics data such as extent of drug absorption, residence time and rate of drug clearance. This can help in sorting out the drugs with unsuitable kinetics at an early stage of the trial and will save money and time (Rudin 2007; Rudin and Weissleder; 2003; Willmann et al. 2008). The SPECT imaging with [^{99m}Tc] is useful for the drugs/molecules, which remained in the circulation for long time. Therefore, delayed imaging is possible to track out distribution at 12 or 24 h. The radionuclides like indium-111 [^{111}In] (half-life – 67.32. h), allows the SPECT imaging follow up for up to 6 days post treatment. Usually antibodies remain in the circulation for a long time; therefore labelling antibodies with [^{99m}Tc], [^{111}In], iodine-123 [^{123}I] and iodine-131 [^{131}I] helps to study distribution over the weeks.

The very common examples of [^{131}I]-labelled antibodies are trastuzumab, tositumomab, humanised anti-CEA and anti-tenascin. The labelled antibody can be used for diagnosis and targeted therapy; the labelling allowed the testing of these tracers at the preclinical levels which can be translated into humans (Akabani et al. 2000; Liersch et al. 2005). Chrastina et al. have demonstrated the role of SPECT imaging in establishing proof of target for recombinant antibody 833c targeting aminopeptidase P (APP) expressed on laminar surface of pulmonary endothelium. Authors have labelled antibody 833c with [^{125}I] and studied biodistribution and pharmacokinetics of the antibody in in vitro cells and the mouse model. The SPECT imaging with [^{125}I]-833c has showed the specific localisation of the antibodies in the lungs and showed maximum uptake at 2 h post-injection. The biodistribution data confirmed the specificity of 833c antibodies for the lungs, and pharmacokinetics showed first-order kinetics with the presence of the significant levels of 833c antibodies in the circulation for up to 30 days post-injection (Chrastina et al. 2010).

Kumar et al. have labelled the anticancer drug doxorubicin with 99mTc and studied its kinetics and tumour detection efficacy in animal models. The pharmacokinetics was studied in rabbits after injecting [99mTc]-doxorubicin intravenously and sampling blood at various time points to plot kinetic of the drug. [99mTc]-doxorubicin was used for detection of tumour in mouse xenografts. After successful preclinical evaluation, the single vial kits were developed, and pharmacokinetics was studied in the human volunteers (Fig. 17.4). The image showed higher uptake of the labelled drug in the lungs, kidneys (clearance route), liver (metabolic site) and spleen at 10 min. On subsequent serial images, the radioactivity of the radiotracer was cleared out from the lungs and minimal at 24 h (Kumar et al. 2015) (Fig. 17.4).

SPECT provide imaging data which can be reported on the basis of visualisation and quantitative analysis. It enables non-invasive evaluation of some drug delivery system like nasal drug delivery distribution in the brain at various time points (Mandlik et al. 2018). The novel drug delivery systems can be validated well using SPECT technology (Fig. 17.5).

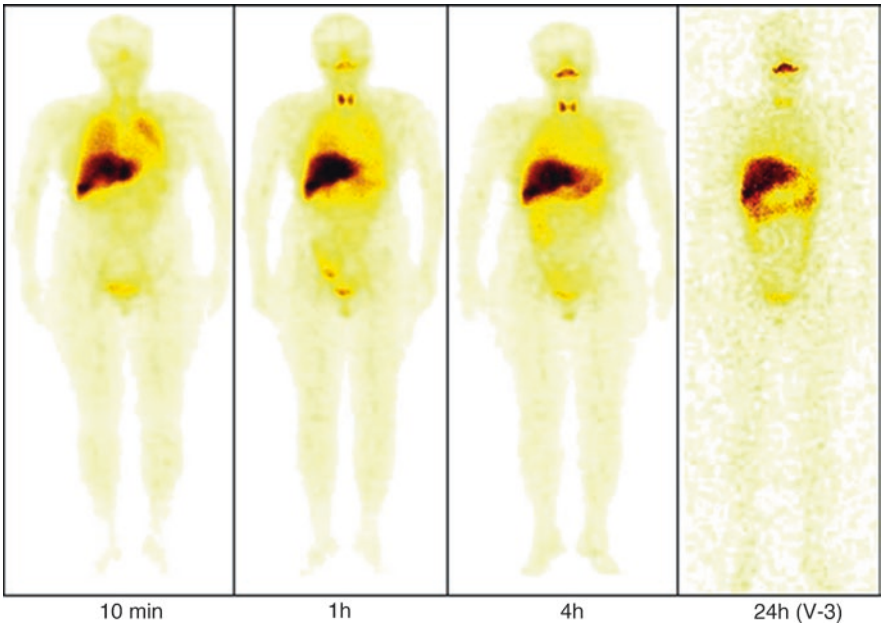

Fig. 17.4 Showing pharmacokinetics and biodistribution of [99mTc]-doxorubicin a normal human volunteer

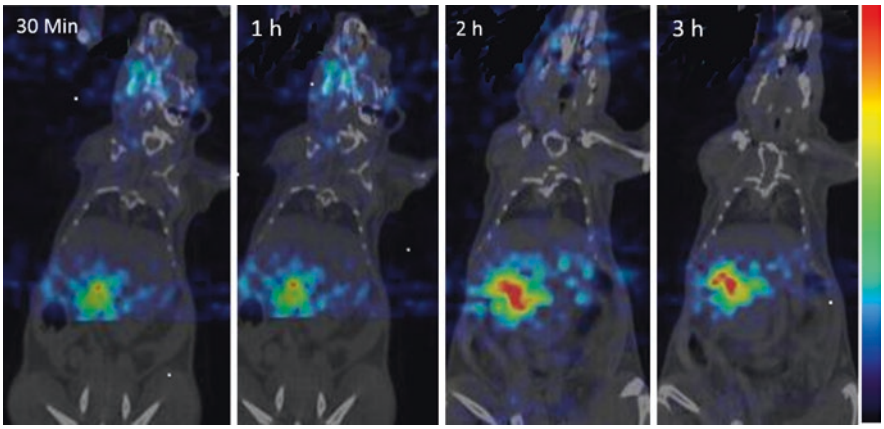

Fig. 17.5 SPECT-CT images of a mouse at different time intervals (30 min, 1 h, 2 h and 3 h respectively), followed by intranasal administration of drug

[^{99m}Tc]-labelled fatty acids analogues have been developed to target cardiac metabolism. SPECT imaging was done to study their biodistribution and kinetics. The affinity and uptake in the cardiac region make them excellent agent for measuring alteration in cardiac metabolism and any alteration in metabolism due to any disease condition (Mathur et al. 2015).

Vaneycken et al. have developed nanobodies which are genetically grafted with antigen-binding loops of NbCEA5. These nanobodies are specific for colon cancer marker carcinoembryonic antigen (CEA). To prove the concept of target, they have labelled nanobodies with [^{99m}Tc] and injected into the mouse LS174T xenografts. The in vivo SPECT imaging has revealed the specificity of the nanobodies for the tumours and renal clearance. The SPECT imaging provides data for tumour-targeting capability of nanobodies, clearance route, biodistribution and blood kinetics (Vaneycken et al. 2010). Radiolabelled drug can be easily traced out on SPECT image, and its distribution can show us whether it will cross blood–brain barrier (BBB) or not. Many drugs failed due to inability to cross blood–brain barrier (BBB). The studies showed 98% of the drugs which target central nervous system (CNS) failed due to their inability to cross BBB (Pardridge 2007).

Receptor-based study is the major part of the drug development. Most of the drugs now have receptor-based mechanism and can be studied with radiopharmaceuticals. Among all the listed radiopharmaceuticals, about 60% are receptor-targeting probes. The drug can be labelled with single photon emitter like [^{99m}Tc] using an indirect approach of labelling in which the labelled molecule not exactly mimics the original molecule, but it is an easy approach to track the tissue distribution and pharmacokinetics of the drug (under development). A variety of SPECT tracers exist for many important targets in oncology and neurology. From the last three decades, many probes have been developed for imaging somatostatin receptors in neuroendocrine tumour cell lines. Many tracers targeting somatostatin receptor have been developed which specifically target SSTR 2 and 5 (Gabriel et al. 2005). The SPECT agents like [^{99m}Tc]-vapreotide (RC-160) and [^{99m}Tc] depreotide (NeoTect) are available and others are still under development, like lanreotide (Rodrigues et al. 2006). Any therapeutic drug targeting SSTR, its therapeutic response can be evaluated by SPECT image using these tracers.

The success of somatostatin receptor imaging has encouraged the radiolabelling of other peptides and has helped to assess the treatment response of the receptor-based therapeutic drugs. [^{99m}Tc]-bombesin used for breast cancer imaging and [^{99m}Tc]-vasoactive intestinal peptide (VIP) can be used to assess the treatment response of drugs targeting VIP receptors (Rao et al. 2001; Shariati et al. 2014). SPECT imaging using [^{99m}Tc] radiolabelled antibodies can be used for microdosing studies, biodistribution and kinetics. Labelling with radionuclide requires an attachment of the bifunctional chelating agent such as DOTA, NOTA or TETA that can conjugate radionuclide like [^{111}In], [^{67}Ga] or [^{67}Cu]. In 2007, National Cancer Institute has conducted a phase zero trial with [^{111}In]-trastuzumab in patients with primary or metastatic breast cancer. The study revealed a correlation between the HER2/neu status of the tumours and trastuzumab uptake and also provided biodistribution, pharmacokinetics and kinetics. The in vitro studies with suitable

cell lines provide data for binding affinity, dissociation constant and inhibition constant. The other advantages are as follows: (1) antibodies can be labelled with radionuclides that emit both beta (therapy) and gamma (diagnostic) radiations like iodine-131 [^{131}I] and lutetium-177 [^{177}Lu], which can be exploited for therapy and (2) the treatment response can be evaluated by imaging. [^{131}I]-tositumomab is such an example as it already has been used for patients with relapsed, CD20+ and follicular non-Hodgkin lymphoma. Similarly, [^{131}I]NP-4 anti-CEA was used for CEA-expressing tumours and [^{131}I] anti-tenascin was used for the treatment of the gliomas (Cheung et al. 2009).

Any new therapeutic drug has to be tested for treatment assessment. The cheaper way to assess the treatment response is SPECT imaging. The tumour volume can be measured by a pretreatment scan using an established agent like [99mTc]-MIBI and a post-therapy scan after completion of the therapy. It will increase the confidence of the study and provide validation to the drug discovery. In combating tumour, most of the drugs in pipeline are targeting the proliferation or growth factors of the cell as proliferation is the hallmark of tumour. SPECT imaging helps in determination of the treatment response of anti-proliferative drugs. The promising SPECT imaging tracer for assessing anti-proliferative therapy is [^{123}I]-labelled L-3-iodo-α-methyl-L-tyrosine (IMT). It is amino acid-based tracer that is based on higher turnover of amino acid metabolism in the tumour cells. In few studies, [^{123}I]-IMT uptake in the tumour site was correlated with proliferation index (Ki-67) in gliomas and soft tissue tumours. These studies provide evidence that IMT can be used a SPECT tracer for evaluating the efficacy of anti-proliferative drugs in the trials (Kuwert et al. 1997; Jager et al. 2000).

The other target for most of drug against carcinoma is inhibiting angiogenesis. Angiogenesis is the process of formation of new vessels and involves a complex sequence of events and express various factors like vascular endothelial growth factor (VEGF). Active tumours have high rate of angiogenesis; hence many drugs target angiogenesis and its factors. VEGF is one of the main targets for anti-angiogenesis drugs. Therefore, there are many SPECT tracers available for targeting and imaging VEGF receptor expression. [^{99m}Tc]-labelled VEGF has been used for evaluating the treatment response of the anti-angiogenesis drugs (Van de Wiele et al. 2002; Bergers and Benjamin 2003). Studies have reported utilisation of [^{99m}Tc] HYNIC-VEGF for the treatment response of the mouse 4 T1 xenograft. They are treated with low dose of cyclophosphamide, and treatment response was assessed by pre- and post-treatment scan. It proves the utility of SPECT agent to validate the treatment response of the new drugs (Blankenberg et al. 2006; Cai et al. 2006).

Angiogenesis expresses another major factor, i.e. $\alpha_v\beta_3$ integrin, which is expressed on the cell surface that mediates endothelial cell migration through basement membrane. Therefore, it is another target of the anticancer drugs. A small tripeptide Arg-Gly-Asp (RGD) has selective affinity for the $\alpha_v\beta_3$ integrin; therefore, RGD was radiolabelled with [^{99m}Tc]. [^{99m}Tc]RGD was proved to be successful tracer to target the αvβ3 integrin receptor expression in the tumours. It was used as a SPECT tracer for evaluating treatment response assessment of drugs targeting $\alpha_v\beta_3$. Several studies have used [^{99m}Tc]RGD in preclinical and clinical subjects for non-invasive imaging

of $\alpha_v\beta_3$ expression. Some studies correlated the extent of $\alpha_v\beta_3$ expression with uptake of [^{99m}Tc]RGD (Fani et al. 2006; Yang et al. 2009). The specificity of the [^{99m}Tc]RGD has been studied by Decristoforo et al. They have proved the specificity of the [^{99m}Tc]RGD in murine xenograft having $\alpha_v\beta_3$-positive and $\alpha_v\beta_3$-negative tumours using SPECT imaging. There was significant difference in the uptake in positive and negative murine models (Decristoforo et al. 2006).

The anticancer drugs induce apoptosis in the tumour cells. For any drug inducing the apoptosis, it can be monitored via using a SPECT. Annexin V is a protein having high affinity for phosphatidylserine expressed on the surface of the cell during early apoptosis. Therefore, annexin V is an identification marker for cell undergoing apoptosis. To use it as a SPECT imaging agent, annexin V has been labelled with [^{99m}Tc]; [^{99m}Tc]-annexin V has been used as a tracer to monitor the treatment response of the drugs causing apoptosis. The pre- and post-treatment scans can assess the therapeutic effect of the drug and provide information about apoptotic status of the tumour (Hajra and Liu 2004; Hajra et al. 2008). Belhocine et al. have tested the feasibility and safety studies with [^{99m}Tc]-annexin V in patients with lung cancer and lymphoma after first course of chemotherapy. [^{99m}Tc]-Annexin V imaging was done before and within 3 days after their first course of chemotherapy. The data showed a correlation between the uptake of the tracer and progression of the disease. Tumour patients with tracer uptake showed complete or partial response to the treatment, whereas patients with no tracer uptake showed progressive disease. It is evident that [^{99m}Tc]-annexin V may be used as a predictor of treatment response in patients with lung cancer and lymphoma (Belhocine et al. 2002). Kartachova et al. (2004) performed [^{99m}Tc]-annexin V SPECT scan in malignant lymphoma patients undergoing radiotherapy before and after the treatment. The uptake of [^{99m}Tc]-annexin V is scaled from nil to high and compared with clinical response. The results showed that complete or partial tumour response was associated with high uptake of [^{99m}Tc]-annexin V during the early phase of treatment as compared to baseline scan and no or low uptake in the case of disease progression (Kartachova et al. 2006).

These studies proved that SPECT imaging can be used for treatment response evaluation effectively and provide substantial evidence to the physician to decide on the therapy course. The therapeutic efficacy of the new drugs can be tested by SPECT imaging that provides a cheaper option than PET imaging. The decision on the drug response to the tumour can be evaluated at early phase of the treatment. Labelling the drug with [^{99m}Tc] provides biodistribution, pharmacokinetics and clearance route information of the drug. The SPECT imaging provides proof of concept and target for the therapeutic drugs.

17.7 Positron Emission Tomography

The source of PET imaging are photons that are generated due to the collision of the emitted positron (from the radionuclide) with an electron, and mutual annihilation leads to the production of a pair of photons at 180°. These photons are detected by

detector as a coincident event. The events are recorded and precise timing enables localisation of the events. The raw data consist of numerous of these coincidence events which are identified as projections. These projections are processed using back projection and iterative reconstruction algorithms to form a number of axial slices. These data are converted to form an image as does in the CT images. To improve the image quality, there has been constant improvements in the detector and image processing. The introduction of the fast scintillation technology in detector material like lutetium oxyorthosilicate (LSO) which reduced the random events and introduction of time of flight (TOF) has contributed to high sensitivity.

The other major advancement in the imaging technology is the fusion of functional and anatomical imaging technology that leads to hybrid imaging modalities like PET/CT and PET/MR. These imaging modalities provide PET and CT or MR images at the same time. PET/MR is the choice of modality for CNS imaging and for paediatric patient as it reduces the radiation exposure by eliminating CT part (Slomka et al. 2016). The common radionuclides used for PET imaging are positron emitter with a short half-life like fluorine-18 [^{18}F] ($t_{1/2}$ = 110 min), carbon-11 [^{11}C] ($t_{1/2}$ = 20 min), gallium-68 [^{68}Ga] ($t_{1/2}$ = 68 min) and copper-64 [^{64}Cu] ($t_{1/2}$ = 12.7 h).

PET is a powerful modality which can provide information about the physiological processes in the organ. Radiopharmaceuticals are injected intravenously which reach the target organs and provide information related to the distribution, kinetics and physiological status. The available PET tracers have high specificity and sensitivity; they are used in picomoles and cause no side effects. PET imaging when combined with CT or MR imaging provides accurate localisation of the tracers on the slice. The modernization in the PET technology has increased the sensitivity and high resolution (~4 mm) for humans (Jung et al. 2016).

The drug can be labelled with positron emitter like [^{18}F] or [^{11}C] and then can be used as a radiotracer. [^{18}F] or [^{11}C] labelling of a compound mimics more closely to the natural structure of the drug as it involves direct covalent bonding to the molecule rather than a chelating binding as in the case of [^{68}Ga or ^{64}Cu]. The quantification software can quantify the labelled drug distribution and tissue uptake. Various types of radiotracers can be used to assess the drug therapeutic response. [^{18}F]-Fluorodeoxyglucose (FDG) is the primarily used radioisotope for oncological applications. It is well established for the diagnosis, staging and monitoring treatment response of many tumours (Fig. 17.6). Apart from their application in oncology, they have wider applications in other areas including neurology, infection imaging, monitoring multidrug resistance and many more (Almuhaideb et al. 2011; Jadvar et al. 2017).

There are many examples in the literature where the therapeutic drugs (ciprofloxacin, doxorubicin, gefitinib, temozolomide) have been labelled with [^{18}F], [^{11}C], [^{68}Ga] or [^{64}Cu] and has been used as a diagnostic tool, studying pharmacokinetics, pharmacodynamics and chemoresistance. Various anticancer drugs like cyclophosphamide (Kesner et al. 2007) and paclitaxel (Kiesewetter et al. 2003) were radiolabelled with [^{18}F], and they were reported to have a promising approach in predicting the response to an appropriate anticancer therapy.

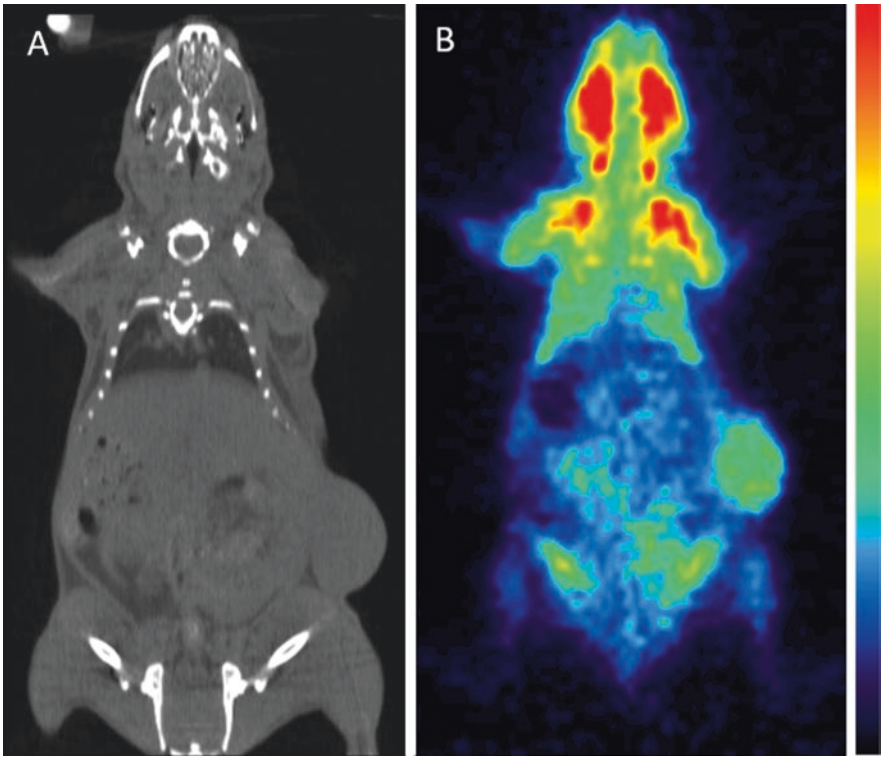

Fig. 17.6 PET and CT images of a mouse showing xenograft tumour in flank region (**a**) and FDG uptake in tumour (**b**)

A number of drugs like cyclophosphamide and paclitaxel have been labelled with SPECT or PET radionuclide ([^{99m}Tc]/[^{18}F]) to detect the stage of cancer and evaluation of chemoresistance at earlier stages. Labelling an anticancer drug helps in predicting its biodistribution, kinetics and pharmacodynamics. Microdosing studies (using 1% of the therapeutic dose) can predict the data for drug absorption, metabolism, excretion and its toxicity (Saleem et al. 2001; Lappin and Garner 2003).

Kiesewetter et al. have labelled anticancer drug paclitaxel (PAC) with [^{18}F]. [^{18}F]-PAC has been used to assess the multidrug resistance (MDR) in the mutant and wild-type mouse models for MDR. [^{18}F]-PAC has been injected into the normal mouse to yield biodistribution data which showed that major uptake was in the kidneys, lungs, heart and blood. To evaluate the in vivo MDR status, [^{18}F]-PAC was injected intravenously into the MDR mutant mouse with mdr1a/1b(−/−) and wild-type mouse. The uptake of [^{18}F]-PAC was 1400 times higher in mutant mouse than in the wild type. It proved the utility of the [^{18}F]-PAC for measuring MDR status in the tumours (Kiesewetter et al. 2003).

Kurdziel et al. have used [^{18}F]-PAC to study the dynamics of the P-glycoprotein (Pgp) pump and MDR effects. They have injected the [^{18}F]-PAC into the three

rhesus monkeys and used the data to evaluate the biodistribution and dosimetry data. They have also studied the FPAC kinetics with or without the effects of the Pgp blocker (XR9576, tariquidar). Their biodistribution data revealed that [^{18}F]-PAC can be administered to humans up to 3 times in a year. The imaging data showed that radioactivity accumulated in the liver and lung after Pgp blocker XR9576 is consistent with Pgp inhibition and demonstrates the potential of [^{18}F]-PAC to evaluate the MDR (Kurdziel et al. 2003).

Hsueh et al. have utilised [^{18}F]-PAC as a chemoresistance predictor in mice bearing human breast (MCF7) tumour xenografts. [^{18}F]-PAC was injected intravenously in athymic nude mice bearing MCF7 and MCF/AdrR (overexpressed Pgp). The mice were imaged and data were deduced for biodistribution and a significant difference was found in the uptake of the [^{18}F]-PAC in xenograft expressing Pgp. Therefore, [^{18}F]-PAC holds promise for the non-invasive estimation of the chemoresistance status and treatment response assessment (Hsueh et al. 2006).

Chemotherapy delivers a combination of single drug to the tumour cells, but with the progression of treatment, the cells develop resistance against the therapy. The resistance may be due to genetic makeup of the cells or acquired with time. There is no tool to assess the in vivo chemoresistance before or during the course of chemotherapy. Therefore, the only way to assess chemoresistance happens when patient came for follow-up after 6 months of treatment and reduction or increment in the size of tumour decides the effectiveness of the chemotherapy. Therefore, it is utmost important to assess the chemoresistance levels before and during the course of treatment. The labelling of drug (which is used for chemotherapy) makes more sense as it will behave in the same manner and shows the real-time interaction between tumour cell and drug. The accumulation of the labelled drug in cells helps in identification of responders and non-responders before or during the course of therapy. Even the behaviour of labelled drug can be tested in vitro on cell lines by having cell line with different genetic makeup and expressions. The uptake in the images is like virtual biopsy targeting the particular receptor in vivo and can be complement to the physical biopsy results. Many studies have showed the non-invasive estimation of MDR status using PET imaging (Hendrikse et al. 1999).

Lacan et al. radiolabelled cyclophosphamide (CP) with [18F] to assess Pgp expression. [18F]-CP was injected into Sprague-Dawley rats for a baseline scan, followed by 2–3 weeks of therapy. After acquiring the images, the mice were sacrificed to study biodistribution. PET imaging showed higher uptake of [18F]-CP; ex vivo studies are also in agreement with imaging data showing higher uptake in the hippocampus and frontal cortex (Goran Lacan 2005).

Apart from assessing Pgp expression, many other expressions can be assessed via PET imaging. The other major class of drugs that target tumour are tyrosine kinase inhibitor, acting on epidermal growth factor receptor (EGFR). Seimbille et al. have reported the synthesis of [18F]-labelled 6,7-disubstituted-4-anilinoquinazolines to image tyrosine kinase receptors non-invasively. The same group radiolabelled gefitinib and erlotinib with [18F] to image EGFR expression. [18F]-gefitinib has been used to image EGFR receptors, to predict in vivo chemoresistance, predicting responders and non-responders for the gefitinib therapy

(Seimbille et al. 2005). Similarly PET tracers like [^{11}C]-PD153035 have been used for quantitative assessment of the EGFR and EGFR TKI overexpression in tumours (Mishani et al. 2008). Temozolomide (TMZ) is a prodrug used for the treatment of the malignant brain tumours. It has been labelled with positron emitter [11C] and has been exploited to study the mechanism of uptake, metabolism and biodistribution of TMZ. A pilot study has been done to evaluate the role of [11C]-TMZ PET imaging to assess the treatment response and mechanism of bevacizumab affecting the blood vessels of the tumour. [11C]-TMZ PET reveals how these changes affect the absorbance of TMZ (Moseley et al. 2012).

These studies proved the role of PET in studying the biodistribution of the labelled drug. It provides the evidence of the target identification. The imaging data give information from clearance of the drug to the target occupancy at the receptor site. In early phase of the drug discovery, dose-ranging studies are required to establish the relationship between plasma concentration and target interaction. PET imaging can play a decisive role in establishing this relation. PET imaging helps in translating the study from preclinical phase to clinical phase. Dosimetry data provide the limit of a dose that can be used in radiolabelled form in a subject per year. The compartmental analysis of the image provides the kinetics of a drug within the tissues and how it is metabolised in the body. The in vivo stability of the drug can be tested by withdrawing serial blood samples at preset time and then analysing by HPLC. The quantification of receptor binding can be done by processing tools, and effect of various factors on the uptake of drug can be evaluated (Berridge and Heald 1999). PET has the capability to assess the treatment response of various therapies; therefore, it may play a role in deciding the optimal dose of the drugs. The biodistribution of a drug may vary with route of administration; therefore, imaging can provide the appropriate route of administration which maximises the uptake at target organ. A common example of a drug administered in aerosol form was deposited into nasal cavity with slow migration towards the lungs. Fischman et al. have administered their formulation via aerosol, but PET imaging showed maximum uptake in the frontal and maxillary sinuses which were away from nasal cavity. This suggested them to use aerosol formulation for sinusitis patients (Fischman et al. 2002).

The specificity of the drugs for the receptors or particular target can be assessed by PET imaging. The labelled drug was co-injected with cold drug which displays the specificity of the drug for the particular receptor by blocking the sites with cold drug. The specificity is an important parameter for psychiatric disorders; as an example, dopamine D2 receptor occupancy shall be around 70% for an antipsychotic effect. The PET imaging can assess the receptor occupancy non-invasively and can be used as a tool for treatment response assessment. PET imaging can provide the pharmacokinetics profiles of the drug, clearance rate, extent of drug absorption and half-life by doing serial PET imaging. These parameters hold much importance in the early phase of drug discovery. The crucial information provided by PET imaging hold an important value as many drugs failed in the later stages due to unsuitable kinetics, poor target occupancy and inappropriate distribution (Hodé et al. 2005).

Saleem et al. have tested pharmacokinetics of antitumor drug N-[2-(dimethylamino)ethyl]acridine-4-carboxamide (DACA) in the first phase trial by labelling the drug with [11C] in five patients. PET imaging with labelled drug was performed to deduce pharmacokinetics, blood flow rate and plasma metabolites. The labelled drug showed rapid clearance from the systemic circulation within 60 min and number of metabolites. From the point of view of toxicity, the labelled drug accumulated very low in the brain and spinal cord.

The data obtained from the study predict the in vivo behaviour of the drug, activity and toxicity of DACA and establish the importance of PET imaging in early phase of drug development (Saleem et al. 2001).

PET has established its role in assessing the therapeutic effect of the drugs. Treatment response for a number of drugs under exploratory clinical trials has been evaluated by PET imaging (Zhu et al. 2011). A number of PET tracers are available to target the hallmarks of the cancer cells like high metabolic rate, amino acid upregulation, higher proliferation rate, hypoxic condition, angiogenesis and apoptosis status (Rice et al. 2011). The low-lying physiological changes in the cell marked by the PET imaging help in diagnosing the disease at earlier stage, and appropriate drug can be used for treatment and treatment response can be evaluated by PET imaging (Chandrani et al. 2017). [18F]-FDG PET was also used for the treatment response assessment of the imatinib mesylate in patients with gastrointestinal tumours 24 h post-therapy. Therefore, the role of 18F-FDG-PET has been established for treatment response assessment for solid tumours (Van Den Abbeele and Badawi 2002). Due to non-specificity of the FDG and uptake in the infection lesions which can lead to false positive results but there are several PET tracers which are highly specific for tumour treatment response assessment (Wu et al. 2013). In view of limitations of the [^{18}F]-FDG, there are PET tracers like [^{11}C]-methionine, [^{18}F]-choline and [^{18}F]-DOPA which are based on amino acid metabolism. These tracers can assess the tumor size and volume more precisely. Drugs targeting amino acid metabolism, their treatment assessment can be directly done by using these agents (Juhász et al. 2014). Also [^{68}Ga] with its well-developed coordination chemistry and cost-effective availability when radiolabelled with fatty acids can be used for metabolic cardiac imaging (Jindal et al. 2014).

There are numerous drugs under development which target proliferation rate of the tumour cell. These drugs mostly inhibit cyclin-dependent kinase or epidermal growth factor receptor. PET has several tracers which can accurately measure the proliferation rate of the tumour, like [^{18}F]-fluorothymidine (FLT). Fluorothymidine is a substrate of the intracellular thymidine kinase and a part of DNA machinery. Therefore, [^{18}F]-FLT is used as a maker for PET imaging to assess the proliferation rate of the tumour in non-small-cell lung cancer patients. Peck et al. have correlated the uptake of [^{18}F]-FLT on the lesions with histological outcome of the lesion biopsy. The proliferation marker on histological sample was evaluated by immunohistochemistry and found in a correlation with uptake of [^{18}F]-FLT. [^{18}F]-FLT has been used for the assessment of treatment response after administration of 5-fluorouracil, and it deduces the mechanism of action of drug at cellular level (Peck et al. 2015).

The effectiveness of any anticancer drug can be measured in terms of the induction of apoptosis at the tumour site. PET imaging can provide information on the induction of apoptosis after the start of therapy. [^{18}F]-annexin V is used as a PET marker for imaging apoptosis status. The study has found a correlation between the uptake of the [^{18}F]-annexin V and apoptosis (Hu et al. 2012).

The drugs targeting angiogenesis (specifically $\alpha_v\beta_3$ integrin) can be monitored by PET imaging. RGD is a peptide which specifically binds to the $\alpha_v\beta_3$ integrin; therefore, [18F]-RGD is a promising agent for imaging angiogenesis (Chen et al. 2004). Niu et al. have labelled a humanised murine antibody cetuximab which specifically binds to $\alpha_v\beta_3$ integrin with [^{64}Cu]. The nude mouse tumour xenografts were treated with cetuximab, and its tumour delivery and distribution of cetuximab were evaluated by [^{64}Cu]-cetuximab. So, labelled antibody has been used for visualising target efficacy and pharmacokinetics of the antibody (Niu et al. 2010).

Hypoxia is another possible reason for the development of resistance to radiotherapy and chemotherapy. Diagnosis of hypoxia at the earliest state helps a clinician to change the course of therapy. Chemotherapeutic agents that belong to class nitroimidazole bind to hypoxic area where intracellular concentration of oxygen is less. Xu et al. have used [18F]-labelled misonidazole as a hypoxic marker in several tumours including non-small-cell cancer and other types, which helps in the monitoring of treatment response (Xu et al. 2017).

Many studies have shown that [^{64}Cu]-, [^{60}Cu]- or [^{62}Cu]-labelled methylthiosemicarbazone (ATSM) has affinity for hypoxic tumours, which has been used to predict therapeutic response in advanced cervical cancer and non-small-cell lung cancer patients. Similarly, [^{18}F]-labelled fluoroazomycin arabinoside (FAZA) and [^{64}Cu]-ATSM have been compared head to head in in vitro cell lines and mouse xenograft models. [^{64}Cu]-ATSM is found to be a better agent than [^{18}F]-FAZA to detect hypoxia in tumours (Kim et al. 2009; Valtorta et al. 2013).

All these studies prove the significant contribution of PET imaging in the drug discovery. It has certain advantages like quantifying drug concentration in tissue, diagnosis, staging and treatment response assessment. Advancement in hybrid imaging has reduced the imaging time and provides anatomical and functional imaging at the same time.

17.8 Optical Imaging

Optical imaging is majorly used for ex vivo and preclinical studies. The principle of optical imaging is based on the emission of light from a substrate after interaction with an enzyme, especially luciferase. The major advantage of optical imaging is that it is radiation-free and multiple animals can be used at the same time. The fluorochromes are excited by a light source and they emit back a light of different wavelength which is detected by a detector. The clinical applications of optical imaging are very limited due to absorption of the light within the tissues. The recent

development in the optic imaging leads to optoacoustic tomography which can image physiological and molecular markers with high resolution. Several optical scanners are available in the market. The main advantage of optical imaging over nuclear medicine imaging is that it does not involve any radioactivity. Reporter gene assay has been demonstrated via optical imaging, which provides information about the action of mechanism of the drug. The drug under investigation can be tagged with fluorescent dye and then injected in animals to localise and establish the drug and target relationship (Graham et al. 2005; Jolly et al. 2005).

Optical imaging can be used in two different approaches: either bioluminescence (BLI) or fluorescence. The major advantages of the optical system are that they are less expensive, easy to operate and cost-effective. This method is well established in drug development for in vitro and preclinical studies. Optical imaging has direct application in assessing treatment response in cell lines (in vitro studies). This method can be used to assess the therapeutic effect in cell line as several cell lines that represent solid tumour types are available. For any therapeutic drug, it is important to monitor change in the tumour volume during the treatment course in animal models. There are numerous studies in which drug pharmacology was tracked in various tumours cell types like brain, breast, lung, sarcomas and multiple myelomas (Cerussi et al. 2010; Gioux et al. 2010). Lee et al. have used bioluminescence imaging to evaluate the treatment response of the various courses of therapy on the D54 glioma cells and on the D54 xenograft mouse models. The bioluminescence imaging results are well correlated with histology (Lee et al. 2007).

Contessa et al. have used bioluminescent reporter engineered probe that targets the N-linked glycosylation (NLG). Non-invasive imaging of NLG showed that the glycan biosynthesis is a novel target for cancer therapy. BLI was used for imaging of in vitro tests and mouse xenograft models (Contessa et al. 2010). Zeng et al. have treated the triple negative breast cancer cell line MDA-MB-231 and their xenograft mouse by combination of rapamycin and cyclophosphamide. The treatment response was studied by BLI and correlated with expression of Ki67, CD34 and HIF-1alpha by immunohistochemistry (Zeng et al. 2010).

BLI has been used for studying pharmacodynamics, in vivo assessment of p-glycoprotein expression and treatment response. Similarly, it can detect bioavailability and tumour drug delivery and in vivo drug concentration.

The optical bioluminescence gives an advantage of studying the interaction with cell lines and detects single cell interaction by using high-resolution fluorescent microscopic techniques like super-resolution microscopy techniques such as stimulated depletion microscopy (STED), stochastic optical reconstruction microscopy (STORM) and photoactivated localisation microscopy (PALM). This gives the advantage of studying the interaction first at cellular level and then at preclinical level in drug development process. Though keeping in mind the advantages of optical imaging, there are several limitations: the depth of the tumour decides the attenuation of the signal and strong signal produced by one organ affects the other organs emitting weaker signal.

17.9 Advanced Imaging: Multimodal Molecular Visualisation of Tumour Angiogenesis by Using a Hybrid Imaging Approach of Ultrasound and Photoacoustics (US-PA)

Jithin Jose

Of late, research is being conducted on the combination of imaging modalities which can provide structural, functional and molecular information in one image with superior sensitivity and spatial resolution. For example in x-ray imaging, especially in digital mammography, the administration of a contrast agent such as iodine with increased vascular residence time is used to enhance contrast from malignant breast lesions due to the presence of high micro-vessel densities in the region (Diekmann and Bick 2007). In ultrasound imaging, power Doppler is used for assessing tumour vascularity (Schroeder et al. 2003). However, the technique suffers from angular dependency and poor signal to noise ratios.

Photoacoustic (PA) imaging is recently developed hybrid imaging approach that combines optical imaging and ultrasound imaging. The method is based on the generation of ultrasound by the absorption of short pulses of laser light in tissue. Since ultrasound propagates through tissue with 2–3 orders lower scattering compared to light, the method has not suffered from limited resolution faced by the optical imaging while still using light as the probing energy. So this fusion imaging allows combining the optical absorption contrast with the high spatial resolution of ultrasound. PA imaging has the potential for identifying both the anatomical features and functional activity of tissues at higher depths. Analogous to optical imaging, multispectral PA imaging can provide information of tumour oxygenation and can serve as a tool for diagnosing malignancy. The method has shown great potential in tumour angiogenesis imaging, whole-body small animal imaging and melanoma visualisation in patients (Li et al. 2017; Jose et al. 2009; Ntziachristos 2010; Wang and Yao 2016; Stoffels et al. 2015; Xu and Wang 2006).

However, the contrast possessed by the tumour in PA imaging is due to enhanced optical absorption from the higher densities of blood vessels present in the tumour region. But in scenarios where tumours with a low density of blood vessels and necrotic areas predominate, PA imaging may not be conclusive requiring other imaging modalities for better diagnosis. Several attempts were made to improve the reliability of PA imaging by integrating other imaging modalities like ultrasound (Needles et al. 2013), X-ray (Huang et al. 2011) and OCT (Liu et al. 2016). Of these, integration of ultrasound imaging is more attractive as there is no need of an extra-hardware and we can also combine the superior imaging features of ultrasound together with PA imaging.

In this section, we will present novel imaging platform of a photoacoustic imaging system integrated with high-resolution ultrasound imaging (US-PA), which can provide multimodal molecular visualisations. Since this is a hybrid imaging approach, the platform offers the real-time imaging of complete anatomical, functional and molecular information non-invasively.

17.10 Ultrasound-Photoacoustic Imaging (US-PA)

Figure 17.7 illustrates the basic principle of photoacoustic imaging, where the laser light is used to illuminate the tissue and the generated ultrasound signals are detected by the ultrasound transducer. In the US-PA imaging, a hybrid ultrasound transducer is used to detect the PA signals generated upon the illumination of nanosecond (ns) laser pulses. The signals are generated due to the thermoelastic expansion where the absorbed optical energy is converted into thermal energy by fast radiationless de-excitation processes of the excited energy levels, called photothermal heating. The resulting temperature rise causes thermal expansion that produces a pressure build-up. The generated PA signals propagate in 3 dimensions with a relatively constant speed of 1.5 mm/μs (average speed of sound in tissues), and the signals can be detected at the surface of the tissue. Since it is the hybrid transducer, the same transducer can emit the ultrasound signals and detect the echoes to perform the ultrasound imaging.

Fig. 17.7 Schematic representation of photoacoustic imaging principle

Ultrasound (US) is a commonly used diagnostic imaging modality in the clinic, and they mainly operate in a frequency range of 2–18 MHz. This frequency range is ideal for deeper tissue imaging (5–10 cm) with the resolution of 200–300 microns. In US imaging, the sound wave is generally produced by the piezoelectric crystal in the transducer. Once produced, the sound wave is directed into the tissues. With the same transducer, the echoes can be detected by switching the system to a transmission/receiver mode. The images of these tissues can be reconstructed by monitoring the time lag of these detected echoes converting the signals into image space (Wagner et al. 2012).

Since the US imaging is based on the detection of reflected echoes, it mainly provides the anatomical information of the tissue. But for the complete understanding of tissue progression and regression, we need more functional and molecular information. Doppler imaging is another approach, which can be combined with the ultrasound imaging as this is also based on the detection of pulse-echo techniques. In the Doppler imaging, the velocity information that mainly arises from the red blood cells in the body is monitored. Depending on the flow of the blood cells, the frequencies of the backscattered ultrasound signals will be shifted to higher/lower frequencies. The magnitude of this shift can be monitored, and thus the functional information such as the velocity of the blood flow in the tissue can be calculated.

In addition to the Doppler imaging, photoacoustic (PA) imaging can also be integrated into the ultrasound systems. Niederhauser et al. were the first to explore this approach integrating the photoacoustic imaging technology with a clinical ultrasound system (Niederhauser 2004). Since the PA imaging is dependent on the optical absorption, it can be used to discriminate endogenous and exogenous signals from the tissues. Furthermore, the vascular oxygen saturation (sO2) and hemoglobin [Hb] concentration can also be monitored by using multi-wavelength PA imaging (Needles et al. 2013).

The resolution of the traditional ultrasound image can be increased by increasing operating frequency. Although there was some technological hurdles to fabricate the high frequency ultrasound transducer arrays, the recent advancement on the machining of the piezoelectric elements enabled the development of ultra-high frequency (15–70 MHz) linear array transducers. All of these technological evolutions and the niche applications of high-frequency ultrasound in cardiovascular, cancer and developmental biology are enlightened in the review article published by Foster et al. (Foster et al. 2011). Although the technology can provide better resolution, one of the trade-offs of this approach is the limited imaging depth. Since the higher-frequency signals attenuate faster than the lower-frequency ultrasound, this imaging approach is ideal for the preclinical imaging where limited penetration depth with higher resolution is required.

Recently, Needles et al. (Needles et al. 2013) developed a platform and integrated high-frequency ultrasound and photoacoustic imaging. The platform uses higher-frequency linear array transducers (9–70 MHz), which can provide the resolution up to 30 microns. The platform is commercially available and it is dedicated

to small animal imaging research. There are also reports on the development of other hybrid imaging systems where they combine lower-frequency ultrasound and photoacoustic imaging by using a specially developed tomographic detector (Mercep et al. 2015). Although technology has diverse applications including clinical research, most of the reported studies are in the field of tumour detection and the development of therapeutic agents. A recent review article from Choi et al. (Choi et al. 2018) provides an excellent overview of all the available US-PA systems and its applications in the translational research.

17.11 Multimodal Imaging of Tumour Angiogenesis

Figure 17.8 illustrates the multimodal imaging features of high-resolution US-PA platform specifically on monitoring the progression and regression of tumour angiogenesis. Since the approach is based on real-time, high-resolution US imaging, tumour cells can be implanted by using the image-guided needle injection. After the implantation, anatomical imaging can be used for the earliest detection of

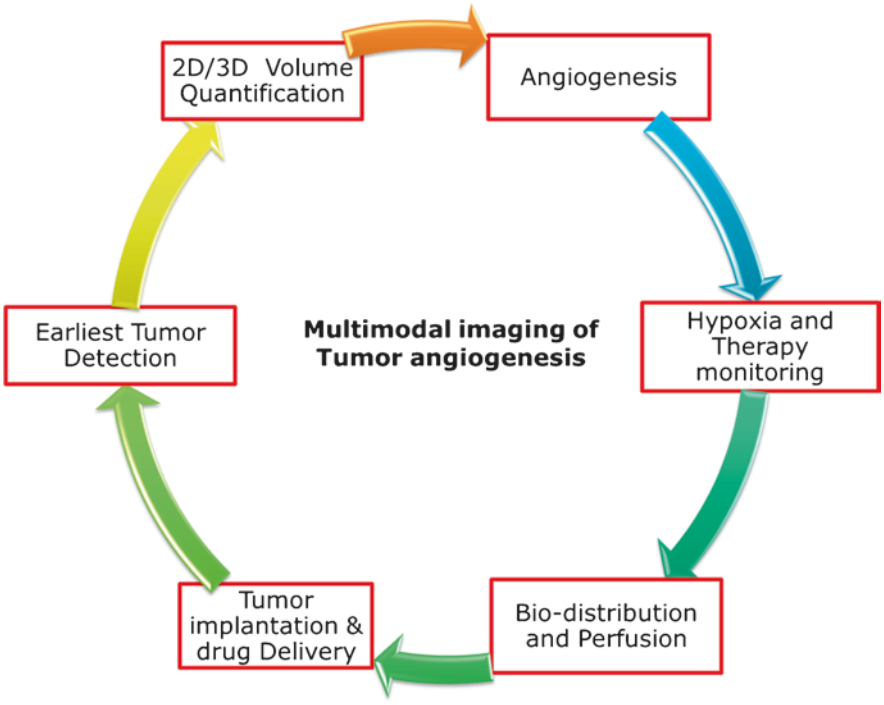

Fig. 17.8 Multimodal imaging of tumour angiogenesis

tumour development. The tumour progression can be monitored using 2D/3D volume quantifications. The functional and molecular information, for example, tumour angiogenesis and hypoxia, can be visualised by the Doppler and PA imaging. Since the platform also allows the full-body scan, the PA imaging can be used to detect and quantify the biodistribution of exogenous tumour-targeting contrast agents.

17.12 Image-Guided Needle Injection (IGNI)

Image-guided needle injection (IGNI) is a procedure that allows the implantation of tumour cells or the extraction of samples from the tissues. In the preclinical cancer research, normally surgery is used to implant the tumour cells or perform the biopsies. But surgical procedures require more recovery time and it is also painful for the animals. With the use of the IGNI, the surgical procedures can be eliminated and the tumour cells can be injected with better precision and reproducibility.

Recently, Huynh et al. (Huynh et al. 2011) used the ultrasound image-guided needle injection approach to induce the orthotopic pancreatic cancer xenograft models in mice. For the evaluation, they also used a highly invasive surgical procedure to implant the tumour cells and later to compare the tumour models developed by both the approaches. Following their study, they reported that the optimised IGNI method prevented the injection complications such as recoil of cells through the injection canal or leakage of cells out of the pancreas into the peritoneal cavity. IGNI approach also offered an improvement over the surgical procedures as the injection procedure took only 30 s versus 5 min and with a shorter recovery and healing time.

Figure 17.9a, b represents the two phases of the IGNI into the left kidney cortex. As step one, the needle tip is visualised in the ultrasound image and positioned with the aid of needle guidance. Later, the needle is inserted into the kidney cortex with a small incision made on the skin by using the same needle prick.

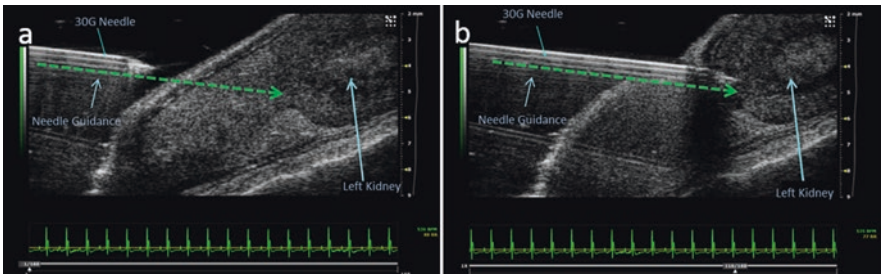

Fig. 17.9 (**a**) Positioning the needle for the guided injection. (**b**) Needle inserted into the left kidney cortex

17.13 Early Tumour Detection and 2D/3D Quantification

After tumour cell implantation, growth can be monitored with the aid of ultra-high-resolution anatomical imaging. With the automated approach, 2D or 3D images of the region of interest can be visualised and its volume can be quantified. For the longitudinal study, the measurement can also be repeated at multiple time points and the tumour growth and its response to therapy can be assessed. Figure 17.10a shows the example of early tumour detection in 2D where the multiple tumour metastases were visualised.

Since the tumour growth is irregular and asymmetrical, for the superior quantification the 3D volume detection can be used, which provides better analysis. A recent study by Pigula et al. (Pigula et al. 2018) assessed the tumour development longitudinally. This study reported that ultrasound enables high-throughput evaluation of orthotopic mouse models via the fast acquisition of 3D tumour images and calculation of volume with a realistic degree of accuracy. Further, they also demonstrated the unique ability of the ultrasound technology on longitudinal treatment to visualise the changes in tumour size as a response to benzoporphyrin derivative photodynamic therapy (BPD-PDT). Figure 17.10b shows the example of such volume quantification where the tumour is imaged in a longitudinal study.

17.14 Tumour Angiogenesis and Vascularity Measurements

Tumour angiogenesis is a large area of cancer research. It refers to the formation of new blood vessels from the existing vessels. There are numerous pathways involved in angiogenesis, which is one of the most commonly referred pathways including vascular endothelial growth factor (VEGF) and its receptors. Since it is related to the new blood vessel formation, the ultrasound Doppler imaging can be used to detect and quantify the vascularity – an index of relative vascular density.

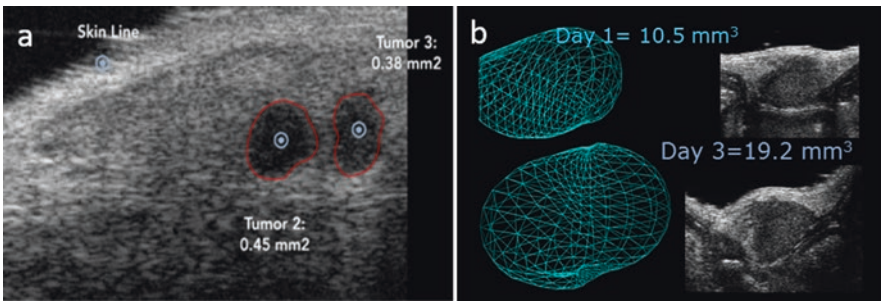

Fig. 17.10 (**a**) Early detection of tumour metastasis. (**b**) 3D volume quantification of tumour on alternate days

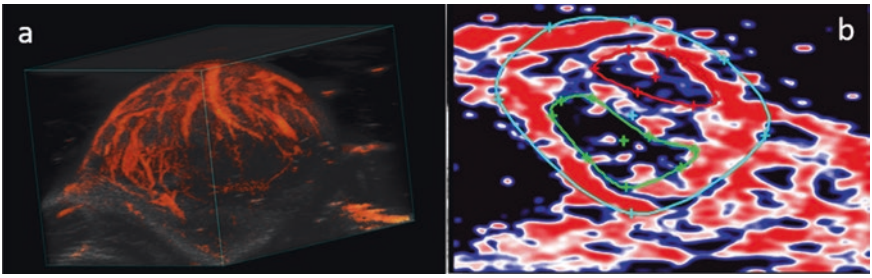

Fig. 17.11 (**a**) 3D Power Doppler image (orange colour) of the tumour overlaid on the ultrasound image (grey). (**b**) 2D parametric map of oxygen saturation where red represents the highly oxygenated regions and blue represents the low oxygenated regions of the tumour

Although there are different types of Doppler imaging modes available (colour Doppler, power Doppler, tissue Doppler, pulse wave Doppler), the most commonly used Doppler imaging mode for cancer research is power Doppler imaging. With the power Doppler imaging, the intensity of blood flow in the vessels can be visualised with superior sensitivity and the percentage of vascularity can be determined. Since this approach can be performed in 2D/3D, the tumour progression and regression or its response to the therapy can be monitored. Figure 17.11a shows the example of 3D Doppler imaging of tumour overlaid on the ultrasound image.

17.15 Tumour Hypoxia and Therapy Monitoring

In addition to the anatomical and functional imaging, the US-PA platform can also facilitate tissue hypoxia imaging in 3D without the aid of any exogenous contrast agents. Although there are methods existing for measuring tissue hypoxia, most of them are superficial or invasive with low temporal/spatial resolution. But in this imaging platform, we can use the photoacoustic imaging to detect the tissue hypoxia non-invasively. Since the optical absorption spectra of oxygenated and deoxygenated haemoglobin are significantly different, multispectral PA imaging can be used to detect the oxygen saturation in tissues and differentiate the oxygen-rich/oxygen-deficient regions non-invasively.

The fundamental aspect of tumour aggressiveness is known to be its hypoxic state. As the tumour grows, it rapidly outgrows its blood supply, leaving regions where the oxygen concentration is significantly lower than in healthy tissues. With PA hypoxia imaging 3D parametric of oxygen saturation of the intact tumour can be visualised. Thus, this approach allows for the real-time monitoring of molecular and functional processes with the added advantage of having the oxygen saturation data fused with high-resolution anatomical images. Figure 17.11b shows the example of a 2D parametric map of oxygen saturation in a murine tumour model. The different

colours in the parametric map embody the oxygen saturation levels as the red colour represents the oxygen-enriched regions and blue denotes the oxygen-deficient regions in the tumour.

Recently, Mallidi et al. (2015) used this platform to predict the response and tumour recurrence in a murine model of glioblastoma, following the photodynamic therapy (PDT). They have used photoacoustic oxygen saturation (StO2) measurement, a surrogate marker for predicting treatment efficacy. They have demonstrated that tumours responding to PDT undergo approximately 85% change in oxygen saturation by 24 h post therapy, while there were no significant changes in oxygen saturation values in the non-responding group. Furthermore, the parametric map of tumour oxygen saturation in 3D can also be used to predict whether the tumour is likely to regrow at a later point of time post therapy. Generally, the probability of tumour regrowth information is not easily available with any of the other imaging modalities, which makes the US-PA platform more attractive to perform early tumour interventions and monitor its efficiency.

17.16 Full-Body Imaging and Biodistribution Studies

Although the US-PA platform can provide the multimodal information of tumour, recent studies have shown that the use of target-specific contrast agents can improve the sensitivity and specificity. Usually, PA imaging uses the inherent contrast afforded by the molecules such as oxy-deoxy-haemoglobin, lipids or melanin in tissues. However, this endogenous contrast is inadequate on several types of tumours where the vascularity is restricted. Thus, this deficiency of the contrast may affect the sensitivity of the PA imaging, which can be addressed by the use of exogenous contrast agents such as near-infrared dyes, gold nanoparticles or porphyrins (Weber et al. 2016).

Figure 17.12a, b shows a full-body image of a mouse before and after the injection of indocyanine green (ICG), an NIR dye. In the image, it is evident that after 6 h the dye is accumulating more in the intestine and it will eventually clear from the body by hepatobiliary route elimination.

Toumia et al. (Toumia et al. 2018) recently developed a dual imaging contrast agent that has responses both in ultrasound and photoacoustic imaging. They have used pristine graphene as a PA contrast agent, which was stably tethered to poly(vinyl alcohol) (PVA)-based microbubbles – a contrast agent used in US imaging. In addition to in vivo whole-body biodistribution imaging of graphene, they also performed multispectral PA imaging to differentiate the endogenous and exogenous contrast from the tissues. From the studies, it is clear that the multimodal imaging platform is ideal for full-body biodistribution of contrast agents and its pharmacokinetics analysis.

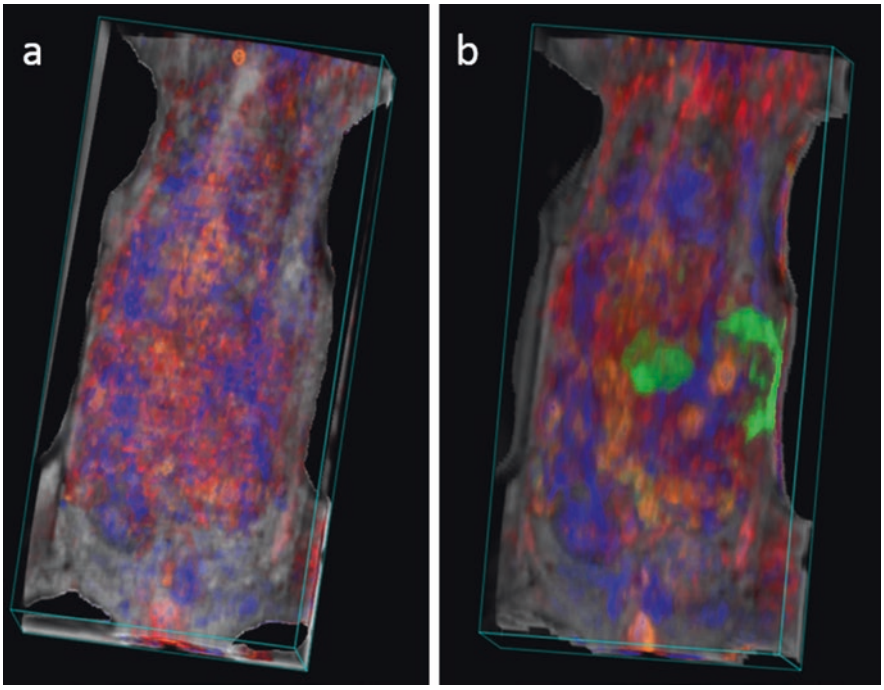

Fig. 17.12 (**a**) Full-body photoacoustic image of the mouse before the injection of ICG: red and blue colours represent endogenous signals arising from the oxy-deoxy-haemoglobin (**b**) after 6 h of injection and green signals represent the accumulation of ICG in the intestine

17.17 Advanced Imaging: MARS and Cancer Imaging

Anthony Butler, Hannah Prebble, Mahdieh Moghiseh and The MARS Collaboration

MARS imaging is an emerging x-ray-based spectral PHOTON COUNTING CT imaging modality that can quantify a variety of materials in a single scan. For cancer imaging, this offers a new approach to characterise and quantify multiple biomarkers, facilitated by labelling with metal-containing nanoparticle-based contrast agents. This functionality sets MARS imaging apart from existing imaging modalities (PET-CT, MRI, dual energy CT) (Anderson et al. 2010b; Moghiseh et al. 2018; Aamir et al. 2014; Anderson and Butler 2014).

The core of the technology is a novel camera (using Medipix 3RX detectors (Ballabriga et al. 2013)) that detects individual x-ray photons and measures their energies, enabling MARS scanners to function as 3D spectroscopes providing an x-ray absorption profile at each point within the sample volume. As each material has a distinct x-ray absorption spectrum, MARS scanners provide material identification and quantification.

Eight counters per pixel register photons if the measured energy exceeds their individual thresholds. This is referred to as single photon counting spectral CT or, more specifically, energy-resolved photon counting CT. The density of multiple biomarkers can be quantified in mg/ml as well as their specific identification without the need for radioactive labels (Moghiseh 2018; Bateman et al. 2013, 2015).

Dual energy CT, the current state of the art in clinical x-ray CT imaging, is sometimes referred to as spectral CT. The two energies are typically used to identify two or three general materials, e.g. a water and iodine map. MARS imaging has many more energies to target more materials with less crosstalk than dual energy CT allowing for accurate quantitative measurements of those materials.

Currently, the only commercially available preclinical system is the MARS small bore scanner. This system is designed for small specimens such as rats, mice and in vitro and ex vivo tissue samples for the purpose of preclinical studies. The applications demonstrated in the following sections were all conducted using a MARS small bore scanner.

This small bore scanner can scan volumes up to 270 mm length and 100 mm diameter. The camera's pixel size is $110 \times 110\ \mu m^2$ allowing the resulting 3D images to typically have cubic voxels of $70 \times 70 \times 70\ \mu m^3$ – standard CT voxel sizes are typically of the order of 500 μm. A resolution at this level means that tumours in mice have been observed with well-defined boundaries (Moghiseh 2018) (Fig. 17.13). With a series of scans, this study could easily be expanded to observe the progression and spread of the tumour, or the reaction of the tumour to some form of treatment.

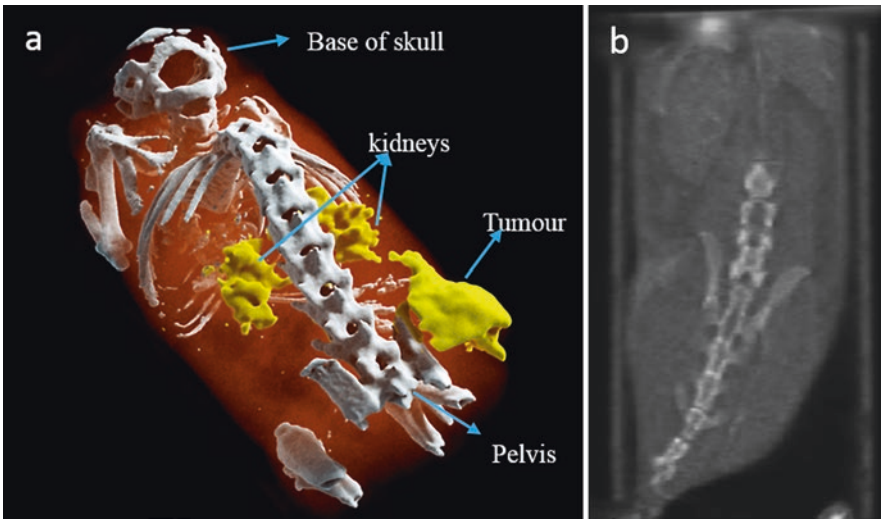

Fig. 17.13 (**a**) Volumetric visualisation of AuNPs (gold) in the kidneys and implanted tumour of a mouse. (**b**) Coronal CT image of mouse containing AuNPs in an implanted tumour, kidneys and spleen. (Moghiseh 2018)

The output from a single scan includes two volumetric datasets. The first represents the effective linear attenuation for a set of energy bins. The energy bins represent non-overlapping regions of the x-ray spectrum stretching from the lowest energy threshold of the counters to the operating voltage (kVp) of the x-ray source. The attenuation data is easily converted to energy-dependent Hounsfield units (HU) and may be studied using existing clinical protocols (Anderson et al. 2010a).

Alongside the attenuation volumes is a set of volumetric maps for a preselected set of materials. The materials could target general anatomy by including canola oil as a representative fat, water as a proxy for muscle and calcium as a proxy for bone. However, they can also include multiple contrast agents rich in different elements, e.g. gold nanoparticles, gadolinium-rich Magnevist and iodine-rich compounds, such as Hexabrix. Such materials may all be included in the same scan. Current studies have demonstrated separation of up to seven different material types (Moghiseh 2018) (Fig. 17.17). For more examples of the quantification of contrast agents, see Panta et al. (2018), Aamir et al. (2014), Ronaldson et al. (2011) and Moghiseh et al. (2018).

The system only detects materials from a preselected list. This means that prior knowledge is required of the materials that are likely to be present. It also means that any material present that is not on the preselected list will be treated as if it is one of those that were selected (typically the most similar material). So to be prudent, a volume representing calcium will be referred to as calcium-like.

Therefore, with prior knowledge of the sample, the most important materials can be targeted to illuminate the presence of substances in tumours and trace those substances over the whole sample. In conjunction with targeted contrast agents, this could be used to map out the shape and size of a tumour, or to track if a drug reaches the intended destination.

In the near future, a human scale system is expected, which is simply a scaled version of the small bore system. This means that any preclinical studies conducted with the small bore system should be translatable to studies at the human scale, thereby offering a direct pathway from preclinical research to clinical trials and onwards into clinical practice. Recently, the first human images were published from a prototype large bore scanner (Giand, August 2018).

MARS offers a unique tool for cancer imaging that is driven by quantifying the presence of multiple materials in a single scan. The results of some of the cancer studies conducted using the MARS small bore scanner are examined below, including the use of uncoated nanoparticles and nanoparticles with a modified surface (known as functionalised nanoparticles) as a novel contrast agent for tumour detection. This is followed by a discussion of the future prospects of this technology in pushing cancer detection and treatment to the next level.

17.17.1 MARS Imaging of Cancer

The properties of both cancer cells and MARS technology are enabling new methods of detecting cancer without the need for invasive biopsies. The goal with any

cancer detection is accurate diagnosis at the earliest stage possible. Recent advances in the cancer field have highlighted complexities in cancer diagnosis, such as heterogeneity of cell phenotypes within tumours. This level of complexity means that a general diagnosis and treatment plan may fail to work for individual patients. Awareness of this issue has strengthened the case for a personalised approach to cancer treatment with diagnosis, therapy and monitoring tailored to the patient.

The need for better diagnosis methods, treatment strategies and patient monitoring is driving the development of new assessment techniques. In addition to research into blood-based biomarkers, non-invasive imaging methods are a key target. Non-invasive methods will only become the tool of choice when they can deliver the necessary information for the clinician to accurately diagnose the patient. This means developing tools that provide not only anatomical information such as the size and location of a tumour but also provide specific quantitative information about the cancer.

17.17.2 Tumour Detection Using Non-functionalised Gold Nanoparticles

A key difficulty of imaging tumours is that they generally need to grow to a reasonable size before it is possible to detect them using standard imaging techniques. On an ordinary CT, a small tumour will closely resemble the surrounding tissue. The obvious answer to this problem is to introduce a contrast agent. For example, in PET-CT, FDG, a glucose analogue with a radioactive fluorine attached, is used to highlight cancer cells that have an increased glucose metabolism. But what if you want to image without using radioactive tracers? This is a question that is being addressed today through the development of nanoparticles for imaging.

Nanoparticles made from high-atomic-number (high-Z) materials are useful contrast agents for x-ray-based cancer imaging for several reasons. Firstly, high-Z materials, like gold, highly attenuate x-rays, meaning they produce a stronger signal compared to low attenuation materials such as fat or water. Other high-Z materials that have been proposed as contrast agents include bismuth, tantalum, hafnium and gadolinium. Secondly, using a nanoparticle structure enables the delivery of a much higher payload of high-Z atoms than an ionic solution would allow. Thirdly, nanoparticles made from gold or other high-Z materials typically have a modifiable surface that can be customised with targeting agents or used for drug delivery. These properties make it possible to image small amounts of material, which can enable detection of features such as small tumours that would typically be indistinguishable from the surrounding materials.

Gold nanoparticles (AuNPs) come in a range of sizes. The size of the nanoparticle will impact where it will end up in the body after injection into the bloodstream. Smaller nanoparticles are quickly filtered out by the kidneys, whereas larger nanoparticles will circulate in the bloodstream for longer. As tumours are growing, they tend to produce new blood vessels in a process known as angiogenesis. These new blood vessels have gaps in the endothelial wall (Cole et al. 2015). Nanoparticles

in the range of 30–200 nm (Albanese et al. 2012) will collect in areas of angiogenesis within a tumour, typically on the periphery, due to a property referred to as enhanced permeability and retention (EPR).

Taking advantage of this effect, AuNPs can be used in conjunction with imaging to highlight tumours with angiogenesis. This has been demonstrated in mice implanted with Lewis lung carcinoma (LL/2) which were injected with 15 nm AuNPs. This size of AuNPs was used to monitor the kidneys as well as tumour angiogenesis (Fig. 17.13a). This figure is a volumetric visualisation of the materials detected within the mouse with the AuNPs in yellow, the bones (calcium-like) in white and the soft tissue-like material in a semi-transparent red colour. This demonstrates how simply the tumour can be identified when materials are used. However, compare this with Fig. 17.13b, which shows a transverse slice of the tumour from the linear-attenuation volume of the same mouse dataset. Despite using the AuNP contrast agent, the tumour is not obvious. This highlights the importance of the material analysis over the simple contrast properties of the AuNP.

Tumour angiogenesis is a useful property to exploit, but individual cancer cells will also take up AuNPs through methods such as pinocytosis or phagocytosis. The amount of uptake can vary depending on the size of the nanoparticle and the cancer cell phenotype or morphology (He et al. 2010; Kulkarni and Feng 2013). Therefore, when designing nanoparticles for cancer imaging, size is an important consideration. This has been demonstrated in the case of OVCAR5 and SKOV3 ovarian cancer cells.

OVCAR5 and SKOV3 cell lines were used to make small pellets that mimicked small tumours. Pellets from each cell line were incubated with four sizes of non-functionalised AuNPs (18, 40, 60 and 80 nm). This was repeated at four concentrations (6.4, 12.8, 25.8 and 38.5 μg/ml). The amount of AuNPs taken up by each cell type was quantified (Fig. 17.14). SKOV3 cells had the highest level of AuNP uptake when the particles were 80 nm, followed by particles of 18 nm. Interestingly, while OVCAR5 cells demonstrated the same uptake trend, the overall quantity of nanoparticles was lower than the SKOV3 cells. Non-functionalised nanoparticles are typically engulfed by the cell which is why the nanoparticle size has a critical role. The next section will explore how the effect of the nanoparticle size can be overcome by using functionalised nanoparticles.

17.17.3 Cell-Specific Imaging with Targeted Gold Nanoparticles

Functionalised nanoparticles can bind to the outside of cells using agents such as antibodies or peptides (Qiao et al. 2009) to target-specific cells (Moros et al. 2012). Nanoparticles offer a high degree of flexibility when it comes to the surface coating and functional groups applied. This means that the techniques described here are applicable to a much wider range of applications than just cancer cell targeting. For example, the same principles could be used to measure the immune response to a cancer treatment. Here, the focus is on using cell surface markers to specifically target a single cancer cell line with gold nanoparticles.

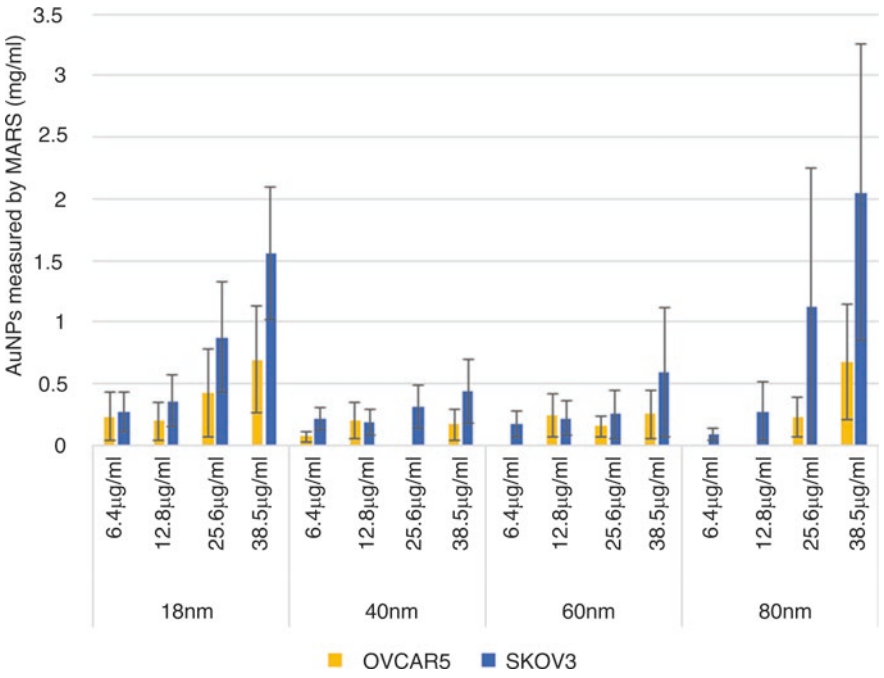

Fig. 17.14 Uptake of AuNPs by OVCAR5 cells (yellow bars) and SKOV3 cells (blue bars) measured by quantitative MARS material decomposition (MD) in mg/ml. Error is SEM

A crossover study was designed to test the ability to target-specific cell phenotypes using Raji B-lymphoma cells (Reddy et al. 2008) and SK-BR3 human breast adenocarcinoma cells (Mahmoodi et al. 2015). Gold nanoparticles were functionalised with rituximab and Herceptin (Moghiseh et al. 2018). Rituximab is a monoclonal antibody that binds to the CD20 antigen overexpressed on the surface of lymphoma cells (Feugier et al. 2005). Herceptin (trastuzumab) is a monoclonal antibody that binds to the human epidermal growth factor receptor 2 (HER2) overexpressed on the surface of SK-BR3 cells. Herceptin is used to treat women diagnosed with HER2-positive metastatic breast cancer (Vogel et al. 2002; Minckwitz et al. 2017). SK-BR3 cells and Raji cells were incubated with the functionalised gold nanoparticles.

Raji cells incubated with rituximab-AuNP showed a high level of gold binding in the material analysis images compared with Raji cells that had been incubated with Herceptin-AuNP (Fig. 17.15). The reverse trend was shown by SK-BR3 cells, where Herceptin-AuNP had a higher binding affinity than rituximab-AuNP (Fig. 17.16).

These results demonstrate the ability to quantify cell specific targeting using gold nanoparticles with the MARS system. Given that antibody-based pharmaceuticals for the treatment of cancer are becoming more common place, the methods described here are likely to be useful not only for identifying the cell phenotype, via targeting specific surface receptors found on cells, but also monitoring drug delivery.

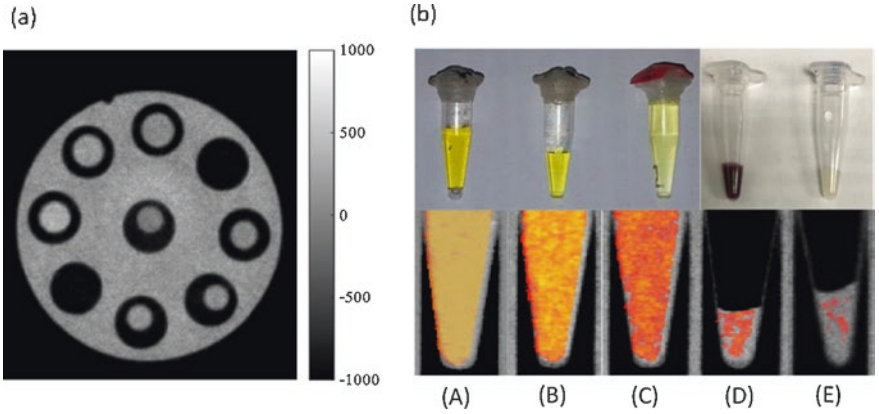

Fig. 17.15 (**a**) CT image of a Raji phantom. (**b**) Photos (top line) and volumetric visualisations of the material analysis (bottom line) of AuCl (A) 8 mg/ml, (B) 4 mg/ml, (C) 2 mg/ml, (D) rituximab-AuNPs bound to Raji cells and (E) Herceptin-AuNPs bound to Raji cells

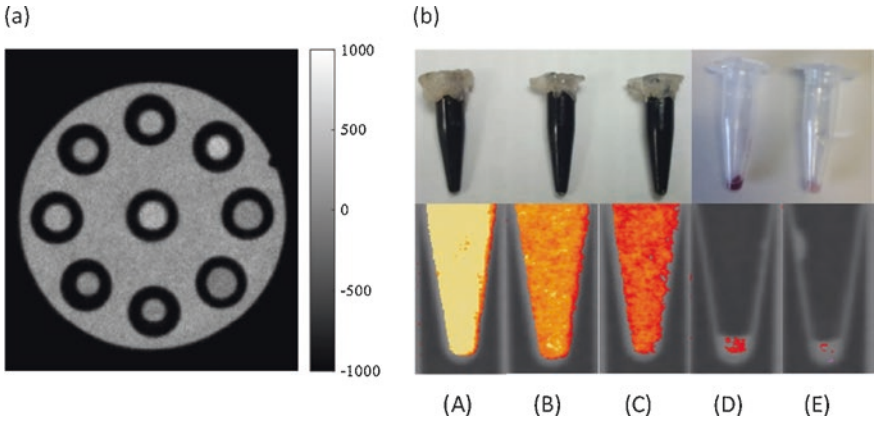

Fig. 17.16 (**a**) CT image of a SK-BR3 phantom. (**b**) Photos (top line) and volumetric visualisations of the material analysis (bottom line) of (A) AuNPs 8 mg/ml, (B) AuNPs 4 mg/ml, (C) AuNPs 2 mg/ml, (D) Herceptin-AuNPs bound to SK-BR3 cells and (E) rituximab-AuNPs bound to SK-BR3 cells

17.17.4 Detecting Microcalcification in a Breast Cancer Model

Microcalcifications are a commonly observed abnormality associated with breast cancer. Breast tissue microcalcifications are thought to be one of the earliest signs of cancer development. There are obvious diagnostic and treatment benefits for being able to accurately identify breast microcalcifications in patients. A group at the University of Notre Dame has been exploring detecting these microcalcifications in mouse models of breast cancer (Curtis and Roeder 2017). They have used MARS imaging and material analysis to see microcalcifications that had been

injected into the flank of a mouse. In addition, they confirmed that the microcalcifications were being accurately measured by using a targeted nanoparticle. The mice were injected with bisphosphonate complexed-AuNPs which could simultaneously be differentiated from the microcalcification in the material analysis images. The advantage to this type of dual approach is that the intrinsic calcium signal can be confirmed using the nanoparticle, a technique that could provide clinicians with useful information for patient diagnosis and treatment planning.

17.18 Future of Imaging

The studies described above have mainly focused on the development of methods using AuNPs as a contrast agent for cancer imaging. However, the potential applications of this technology are much more wide ranging.

As mentioned earlier, various high-Z materials have been proposed as candidates for use as nanoparticles in x-ray imaging including hafnium, yttrium and bismuth. As different materials offer different chemical and physical properties, the range of applications of nanoparticles as both a contrast agent and treatment delivery tool is likely to continue to increase. An example of this would be targeting functionalised nanoparticles to the site of a tumour. Once there, imaging can confirm there is a high enough density of nanoparticles present before they are 'activated' via radiation or ultrasound, producing a localised heat reaction to kill off cells or releasing a toxic treatment. The ability to confirm drug dose prior to activating the treatment will help to ensure a successful result.

The applications detailed so far use a single contrast agent, but, unlike PET or SPECT imaging, MARS imaging is not limited to a single contrast agent per scan. The high material specificity allows multiple contrast agents to be detected in a biological sample simultaneously, across both the whole object (Fig. 17.17) and within a single voxel. The possibility of detecting mixtures of contrast agents creates the potential to answer much more subtle questions using imaging. Functionalised AuNPs could be used to detect HER2-positive cells as demonstrated earlier, while simultaneously functionalised hafnium nanoparticles could be added to image another cancer cell phenotype. This kind of information may allow clinicians to tailor their treatment plan based on the mixture of cell phenotypes present. The area where this is likely to have the largest impact is for small, non-solid tumours, which are typically difficult to image. The ability to measure different contrast materials may enable clinicians to follow treatment progress over time.

The high spatial resolution of this modality combined with material analysis suggests that it will be beneficial for tumour edge detection. This may be through the use of nanoparticles or contrast agents to detect the tumour, or through analysis of changes in lipid content between tissues. Figure 17.18 shows the level of definition between areas with contrast and those without.

Lipid content analysis may also be useful for applications, such as looking at changes in the liver, or for measuring the density of breast tissue (Fig. 17.19). This information may be particularly useful for radiotherapy where calculating the dose required needs to take into account the composition of the surrounding tissue.

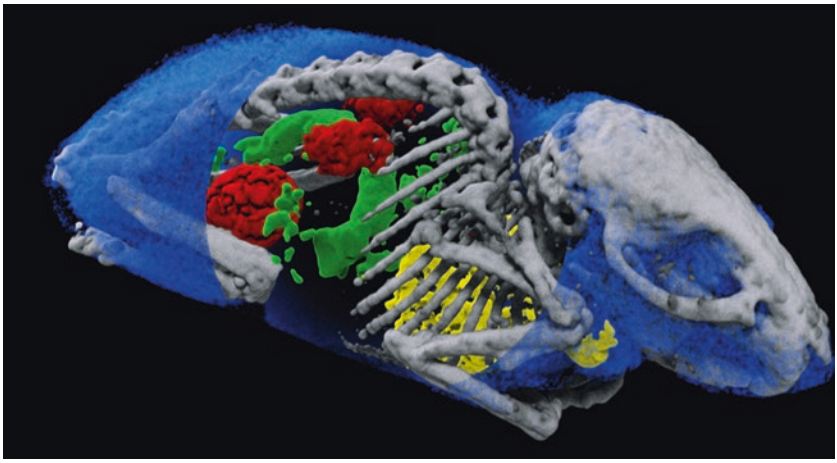

Fig. 17.17 Volumetric visualisation of a mouse with AuNPs (yellow) in the lungs, iodine (red) in the bladder and kidneys, gadolinium (green) in the digestive system and bone (white)

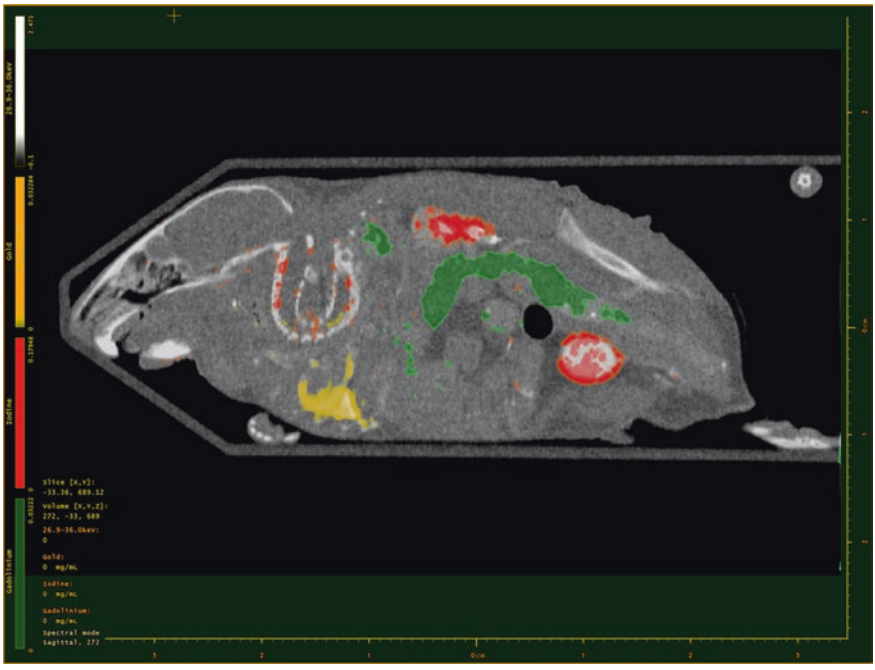

Fig. 17.18 Sagittal slice view of a mouse with AuNPs (yellow) in the lungs, iodine (red) in the bladder and kidneys, gadolinium (green) in the digestive system

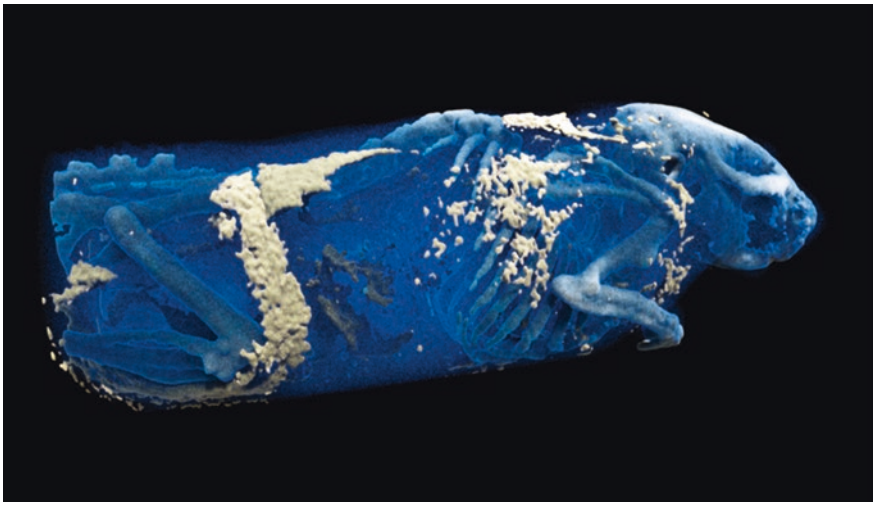

Fig. 17.19 Volumetric visualisation of an APoE−/− mouse without contrast with lipid displayed in beige, soft tissue in blue and bones in white

17.19 Future Perspective

Molecular imaging has accelerated the drug discovery as it is consistently applying for preclinical phases for studying pharmacokinetics, pharmacodynamics and assessing treatment responses. The imaging data provides useful information about the biodistribution and target tissue or organ of the drug. Though molecular imaging is increasingly being applied in drug development, still, there are many challenges that need to be addressed. The advancements in the imaging systems and processing software increased the use of imaging in drug development process. Cancer drug development is expanding at rapid rate and requires enormous resources. The approval rate is low and put great financial burden on the companies. Molecular imaging can fasten the process and provide valuable data at an early stage to decide the pharma companies whether to continue with same drug or not. The limitation of the molecular imaging is being an expensive technology, but the data it provides has valuable and in-depth information. The imaging data in a combination with other data like tumour biopsies, circulating tumour DNA or confirming with genomics and proteomics give a complete picture of the effectiveness of the drug. Imaging technology gives an option of non-invasive way to measure the pharmacological properties and therapeutic efficacy in early stages of drug discovery by using a

minimal quantity of the drug. PET/CT or PET/MR is useful in the assessment of therapeutic response of new drug in early phase clinical trial.

The lack of standardisation of image acquisition protocols and data analysis, a deficient validation of biomarkers and the lack of specific imaging probes for some molecular targets are the critical points which need to be addressed. Solving these issues will increase the role of imaging in drug development process and increase confidence in decision-making. In spite of having these limitations, molecular imaging is being integrated into the pharmaceutical industry infrastructure that will reduce the cost and time for new drug development.

This is a unique platform, based on hybrid imaging of ultrasound and photoacoustic (US-PA) imaging. The platform combines the optical and ultrasound imaging, which has the advantage of obtaining multimodal information in 3D to have a better exploration of small animals. Consequently, this platform is ideal for the tumour angiogenesis studies where we can aid the image-guided needle injection (IGNI) to implant the tumour cells on the specific region with precision and reproducibility. The high-resolution ultrasound imaging can be used for the early detection of tumour growth and subsequent volume changes while performing the longitudinal studies. In addition to the above anatomical information, the vascularity of the blood vessels and the respective oxygen saturation levels can be visualised by using the Doppler and photoacoustic imaging. Since we can detect real-time tumour angiogenesis and the hypoxic tumour regions beyond the use of any exogenous contrast agent, the platform will be ideal for the multimodal imaging and visualising the effect of anticancer drugs.

Although the platform is multimodal and provides a complete analysis of tumour angiogenesis, it can be expedited to the further dimension by integrating a camera which can facilitate optical imaging. Recently Scheepbouwer et al. (Scheepbouwer et al. 2016) performed longitudinal studies of murine orthotopic bladder tumour and performed multimodal imaging of US-PA. They also performed bioluminescence imaging (BLI), an optical imaging approach concurrent to the US-PA imaging. In the analysis, they reported that each modality was able to provide complementary information on tumour growth and its kinetics. Optical imaging is cost-effective imaging modality and it is highly sensitive to assess the in vivo tumour growth. Although optical imaging has the limitation on resolution and the depth information, the technology is easy to use and can provide rapid assessment of the location of the tumour in the whole body of the small animals. So the combination of an optical imaging setup to the US-PA platform can act as a navigation tool to localise the tumour and then provide additional details of tumour development by using the US-PA platform. In general, this approach will allow the integration of individual strengths of each modality to enable the sensitive and improved quantification of tumour angiogenesis.

MARS imaging has the capability to change the future of cancer diagnosis, planning and implementation of treatment and patient monitoring. This is achieved by the imaging and quantification of tissue components, biomarker labels and pharmaceuticals to monitor drug delivery and treatment effectiveness. A small glimpse into this future has been demonstrated here in preclinical studies using gold

nanoparticles as a contrast agent for cancer imaging. The ability to use the properties of the cancer cells and tissues to improve diagnosis ease and accuracy is helpful in establishing new methodologies for cancer diagnosis.

Acknowledgements for Sect. 17.17

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Genomic Applications and Insights in Unravelling Cancer Signalling Pathways

18

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Abstract

The human genome project and subsequent deluge of various high-throughput data in all aspects of molecular biology has not only augmented our knowledge at a breakneck speed but also shifted thinking paradigms about understanding and management of human health and disease. Whole genome sequencing is quickly becoming the first step of disease management particularly in the case of cancer. In this chapter, we have discussed recent advancement in the knowledge in cancer biology through genomic approaches – with a focus on signalling pathways.

Keywords

Genomics · CNVs · Chromothripsis · Hi-C · miRNA · WGS · Precision Medicine · Cancer · Single nucleotide variations · Epigenomics · Candidate genes · Transcriptomics · Cancer therapy

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18.1 Cancer as an Extreme of Health-Disease Continuum

From the moment a single cell starts to live, it also starts to die. Between the birth and death, life tries to maintain equilibrium. This is true for all living cells and organisms. For the entire life span of a cell, or functioning units made up of living cells, there is a constant adjustment to retain equilibrium against many odds. Almost every time when the cellular machinery adjusts to retain or regain the equilibrium, it "pays a price" that is a trade-off for survival fitness. Usually, the trade-off manifests itself in a phenotypic change which is categorized as a disease. Under a dynamic equilibrium, such phenotypic changes are chronic and do not drastically alter the fitness of the cells or the whole organism. In an imaginary scale of mortality where zero implies *not fit for life* and one implies immortality, it is important that the living systems remain dynamic between the extremes.

Cancer is a condition where this dynamic equilibrium shifts and the living systems get closer to one in the scale mentioned above. Ironically, when the cells as units of living systems become robust (immortal), the whole organism becomes unstable as the equilibrium becomes irreversibly perturbed.

This understanding of cancer being one extreme of the health-disease continuum has enabled us to use our knowledge of healthy systems in cancer as well as apply the knowledge of cancer to understand healthy systems. The advances in the area of genomics and high-throughput sequencing have accelerated this process at an exponential pace.

Below we have discussed the different genomic approaches used to decipher various signalling pathways in cancer towards better disease management and therapy. In Fig. 18.1, we have provided an overview of the various genomic approaches that are discussed in the following pages.

18.2 Cancer in the Postgenomic Era

The decision to sequence the entire human genome in the last decade of the twentieth century is one of the biggest game-changing strategies in biomedical research and disease management. Even the proponents of that project could not have envisaged that within two decades of the first announcement of the draft human genome, genomics will invade into almost all areas of biological research and beyond. Now an entire human genome can be sequenced in less than 24 h and at a much lower cost than previously imagined. The dynamic nature of the health-disease continuum portrayed earlier is also applicable to the DNA or the genomic level information. Primarily driven by the genomic technologies, we have now learnt that even two cells of the same or different origin in the same individual's brain can have different genome sequences with or without physiological consequences (Cai et al. 2015; Sharma et al. 2016). The genome dynamics is also applicable between the normal and the cancer genomes. In the beginning of the genomic era, the individual genomes of Craig Venter and James Watson were published along with an individual with cancer (acute myeloid leukaemia). Surprisingly, the majority of the single

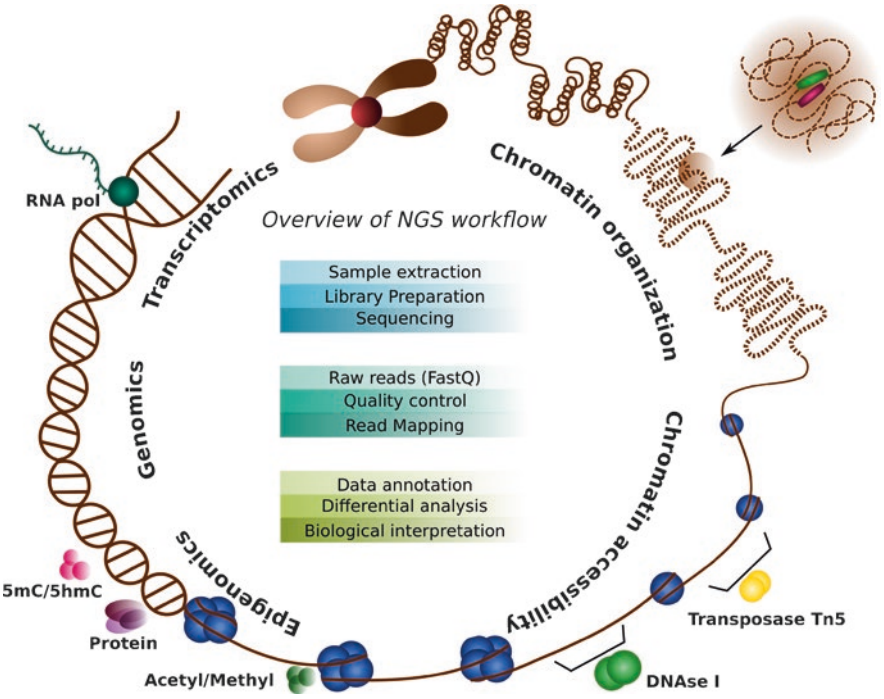

Fig. 18.1 Overview of genomic applications in cancer signalling. NGS refers to next-generation sequencing using massive parallel sequencing technologies that allow unprecedented profiling of different "omics"-level information. Chromatin organization and accessibility, epigenome, genome and transcriptome are the different "omics" layers that together can determine phenotypic expression in cancer. The figure gives an overview of the experimental and analysis methods

nucleotide variations were shared between the "normal" and the "cancer" genomes. This discovery and many more subsequent genome analysis has implicated the arbitrary nature of definition of a *normal genome* based on sequence data alone (Hesketh 2013). This dynamicity or fluidity of the genome has only become more and more profound as we added thousands of individual genome sequences in the database. However, there are genomic variations that are definitely primary driver mutations or associated with cancer and its signalling pathways.

18.2.1 Genomic Approaches to Understand Cancer Signalling

The human genome exhibits a variety of genetic alterations like single nucleotide variations (SNVs) including missense and nonsense mutations, insertions and deletions (in-dels), copy number variations (CNVs) and chromosomal rearrangements like inversions and translocations (Freeman et al. 2006; International HapMap 2005; Xi et al. 2010). Cancer, being a "disease of genome", can carry aberrations for any one or multiple of these subtypes that promote genome instability (Chakravarthi

et al. 2016; Stratton et al. 2009). Now, genome and whole exome profiling of tumour including single cells allow for unbiased identification of novel cancer risk-associated genomic variations and unravel genes and genetic pathways that are impossible to decipher by a reverse genetics approach. This has paved way for development of large consortia efforts such as The Cancer Genome Atlas (TCGA) (https://cancergenome.nih.gov/) and International Cancer Genome Consortium (https://icgc.org/) that further enhances our understanding of the disease. TCGA has used somatic mutations found from exome sequencing data and other "omics" (RNA-seq, DNA methylation, DNA copy number alterations) datasets to analyse mechanisms and patterns in ten canonical pathways namely, cell cycle, Hippo, Myc, Notch, Nrf2, PI3-kinase/Akt, RTK-RAS, TGFβ signalling, p53 and β-catenin/Wnt. They have found that 89% tumours have at least one driver or primary genetic alteration in these pathways, with 57% tumours having at least one alteration targetable by currently available drugs. About 30% of tumours were found to have multiple drug targetable aberrations, opening avenues for combination therapy (Sanchez-Vega et al. 2018). Below we have further discussed a fraction of the available literature to provide a glimpse of the state of the art of genomics in unravelling cancer signalling.

18.2.2 Single Nucleotide Variations (SNVs)

SNVs arise in the DNA sequence due to DNA replication errors – which are random in nature. For nuclear genome of human cells, an efficient DNA repair mechanism corrects most such errors. However, in cancer, the repair mechanism is usually compromised, resulting in accumulation of many SNVs in the genome. Whole exome sequencing has identified mutations in MYC-associated factor X gene (*MAX*) in hereditary pheochromocytoma (PCC). Further analysis confirmed lack of functional MAX in rat PCC cell line indicating involvement of MAX in progression and development of neural crest cell tumours (Comino-Mendez et al. 2011). WGS in multiple myeloma samples identified recurrent mutations in cis-regulatory elements (CRE) of *PAX5* (Paired Box 5). This study also utilized whole exome sequencing (WES) to identify five significant mutations in coding and non-coding regions of the genome associated with signalling pathways like mitogen-activated protein kinase (MAPK), NF-kB, cytokine and G protein-coupled receptor (GPCR) signalling (Hoang et al. 2018). Genome-wide association studies (GWAS) of breast cancer revealed enrichment of risk-associated SNPs in binding sites of *FOXA1* (forkhead box A1) and *ESR1* (estrogen receptor 1) (Cowper-Sal lari et al. 2012). FOXA1 is a critical transcription factor in ESR1-positive breast cancer cells (Hurtado et al. 2011; Laganiere et al. 2005; Lupien et al. 2008). Whole exome and targeted sequencing of colitis-associated cancer (CAC) patients identified 11% of the cancers harbouring somatic mutation in *RNF43* (Ring Finger Protein 43), which was also associated with increased expression of *c-Myc* (Fujita et al. 2018). An amplicon-based next-generation sequencing in 106 primary neuroblastoma tumours identified TIAM1 (T cell lymphoma invasion and metastasis 1) variants and was associated with better

disease-free survival. The variations were distributed in the N-terminus region, which is required for MYC binding. This resulted in inhibition of apoptosis leading to neuritogenesis (Sanmartin et al. 2017). Whole exome sequencing (WES) of six follicular thyroid cancer cell lines revealed recurrent mutations in genes in the RAS/ERK1–2/AKT and CDK1/cyclinB signalling pathway that can be therapeutic targets (Erinjeri et al. 2018). WES of cell-free DNA from RAS/BRAF/PIK3CA wild-type colorectal cancer patients with primary resistance to VEGF:VEGFR2 pathway found somatically mutated VEGFR2 as modulators of antiangiogenic therapies (Toledo et al. 2018). Targeted amplicon sequencing in primary central nervous system lymphoma tumours revealed frequent mutations in PIM1 (Pim-1 proto-oncogene), MYD88 (myeloid differentiation primary response 88), ERBB3 (Erb-B2 receptor tyrosine kinase 3) and somatic activations in RTK-RAS-MARK signalling and aberrations in PTEN-PI3K-AKT pathways (Takashima et al. 2018). WES of natural killer cell leukaemia (ANKL) patients revealed mutations in *STAT3* (signal transducer and activator of transcription 3) (21%) and RAS-MAPK pathways (21%). Following these leads, by drug sensitivity profiling, NK cells were found to be highly sensitive to JAK and BCL2 inhibition compared to other hematopoietic cell lineages (Dufva et al. 2018). In anaplastic lymphoma kinase-positive, crizotinib-resistant, non-small cell lung cancer patients, multiple somatic mutations (89 relevant somatic mutations in 74 genes) were identified in proteoglycans in cancer-related pathways that influence epithelial to mesenchymal transition (EMT) (Wei et al. 2018). In Fig. 18.2, a schematic representation of the proteoglycan-mediated pathways in cancer is provided. The genes highlighted in the figure are identified by different genomic techniques and discussed in multiple places within this chapter.

WES of metastatic clear cell renal cell carcinoma (ccRCC) from 35 cases revealed association of clinical benefit to loss-of-function mutations in PBRM1 (polybromo 1) gene, which encodes a subunit of the PBAF SWI/SNF chromatin remodelling complex (Miao et al. 2018). They validated this finding in an independent cohort of 63 ccRCC patients treated with PD-1 or PD-L1 (PD-1 ligand) blockade therapy alone or in combination with anti-CTLA-4 (cytotoxic T lymphocyte-associated protein 4) therapies. They suggest that PBRM1 loss in ccRCC may change global gene expression profile in cancer, altering response to immune checkpoint therapies.

18.2.3 Copy Number Variations (CNVs)

In addition to SNVs, genomes are also prone for large-scale rearrangements affecting the relative positioning of genes and regulatory regions. These are copy number variations (CNVs). CNVs are more dynamic than SNVs and can affect larger regions of the genome. In cancer due to general instability of the genome occurrence of CNVs with or without a physiological relevance is quite frequent.

Copy number analysis in CRC samples revealed amplifications of *IGF2* (insulin-like growth factor 2), *KRAS* (KRAS proto-oncogene, GTPase) and *MYC* and deletions of *FHIT* (fragile histidine triad), PTEN and SMAD (Seshagiri et al. 2012).

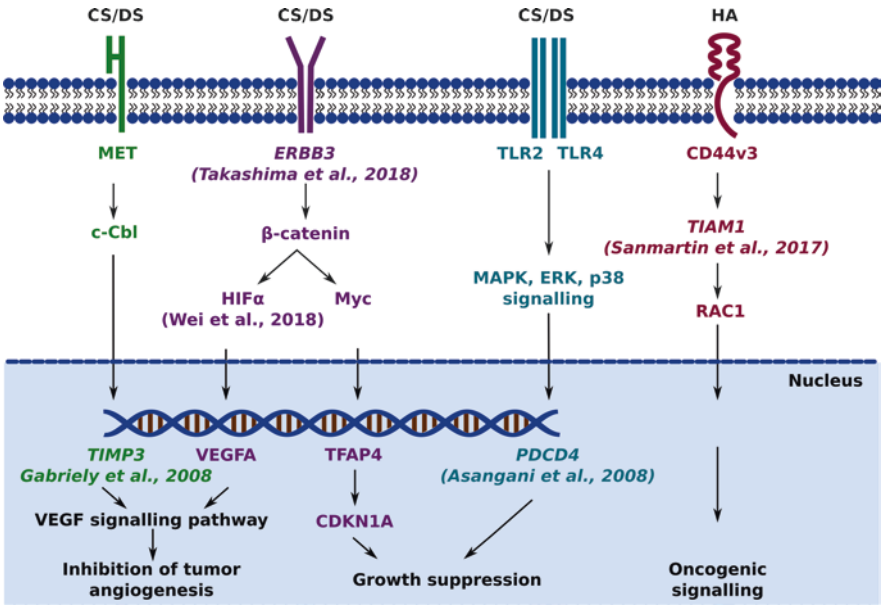

Fig. 18.2 Proteoglycan-mediated cancer signalling unravelled by genomic applications. Proteoglycans (PGs) like hyaluronan (HA), which does not occur as a PG but exist in free form, chondroitin sulfate proteoglycans (CSPGs) and dermatan sulfate proteoglycans (DSPG) contribute to cancer-associated pathways including proliferation, angiogenesis and/or metastasis, affecting tumour growth and progression. The figure gives an overview of proteoglycan-mediated signalling in cancer cells. The genes highlighted in the figure are identified by different genomic techniques and discussed in details in relevant sections of the chapter. For further details of the upstream mechanism refer to KEGG pathway hsa05205. (https://www.genome.jp/kegg-bin/show_pathway?map=hsa05205&show_description=show)

WGS identified multiple CNVs of CREs including deletion of five CREs interacting with *MYC* promoter and were associated with higher MYC expression in multiple myeloma samples (Hoang et al. 2018). Copy number analysis of 417 clear cell renal cell carcinoma (ccRCC) identified loss of chromosome 3p in 91% of the samples, deletions of 9p21 and 10q23 regions harbouring tumour suppressor genes *CDKN2A* (cyclin-dependent kinase inhibitor 2A) and *PTEN*, respectively. Focal amplifications of chromosomal arms were also identified and included genes like *PRKC1* (protein kinase C iota), *MDM4* (mouse double minute 4), *MYC* and *JAK2* (Janus kinase 2) (Cancer Genome Atlas Research 2013). *CCND1* gene amplifications were found to occur at a frequency of 4.8%, whereas *CCND1* single nucleotide mutations had low frequency in several cancers (Xu and Lin 2018). This study revealed that C-terminal CCND1 mutations occurred most commonly in endometrial adenocarcinomas and are putative driver mutations, resulting in nuclear retention and protein overexpression. Somatic variation analysis of WES data from tumour samples from both ovaries and peritoneal metastasis of high-grade serous ovarian cancer (HGSOC) patients identified clonal lineage within left adnexa, revealing loss of heterozygosity

in chromosome 17 containing TP53, NF1 (neurofibromin 1) and BRCA1 (breast cancer type 1 susceptibility protein) mutations (Norris et al. 2018). Their results suggest that HGSOC patients have early and biallelic inactivation of NF1 and gave insights into potential targeting of RAS signalling in NF1 patients. WES of ANKL patients revealed JAK-STAT copy gains, and integrating public domain data it was observed that these alterations are recurrent also in extra nodal NK/T cell lymphoma, nasal-type (NKTCL) samples (Dufva et al. 2018). WES of ASCC samples identified chromosome 3q gains (affecting PIK3CA) and loss of chromosome 11q (altering ATM) as the most common CNVs (Cacheux et al. 2018). Comparative genomic hybridization in multiple myeloma (MM) samples revealed gain of chromosome Xp11.3 harbouring miRNA-221–222 in dexamethasone-induced drug resistance MM samples but not in sensitive MM cells. PUMA (P53 upregulated modulator of apoptosis) is a direct target of miRNA-221–222 and significantly reduced expression of PUMA was found in resistant compared to the sensitive cells (Zhao et al. 2015).

18.2.4 Gene Fusions

The first gene fusion identified was between breakpoint cluster region (BCR) and the second exon of Abelson murine leukaemia viral oncogene homolog 1 (*ABL1*) gene resulting in an abnormal tyrosine kinase and was detected in 95% of chronic myelogenous leukaemia (CML) patients (Dreazen et al. 1987; Rowley 1973). Multiple fusions were detected subsequently by cytogenetic analysis (Larson et al. 1984; Parker and Zhang 2013; Turc-Carel et al. 1987). The advent of NGS allowed parallel detection of multiple fusion events in cancers. WGS and RNA-seq analysis of colorectal cancer (CRC) samples identified two rearrangements – involving known activators of Wnt signalling, RSPO2 and RSPO3 (R-spondin family members) (Seshagiri et al. 2012; Yoon and Lee 2012). The first fusion involved exon 1 eukaryotic translation initiation factor 3 subunit E (EIF3E) with exon 2 of RSPO2. The identified fusion partner for RSPO3 was PTPRK (protein tyrosine phosphatase, receptor-type K). Additionally, overexpressing the fusion proteins led to activation of Wnt-responsive luciferase reporter (Seshagiri et al. 2012).

18.2.5 Other Complex Genomic Rearrangements in Cancer

SNVs, CNVs and gene fusions are present in both normal and cancer cells where the cancer cells present these events more frequently. There are other types of genomic rearrangements, which are unique to cancer cells. These events are collectively called chromoanagenesis (Pihan 2013). The discovery of these events was not possible without the unbiased nature of whole genome sequencing and mapping. It is observed that in approximately 3–5% of all cancers, the driving mechanism is shattering of one or more chromosomes into many pieces, thereby disrupting the genomic integrity and stability. Such shattering events trigger DNA repair

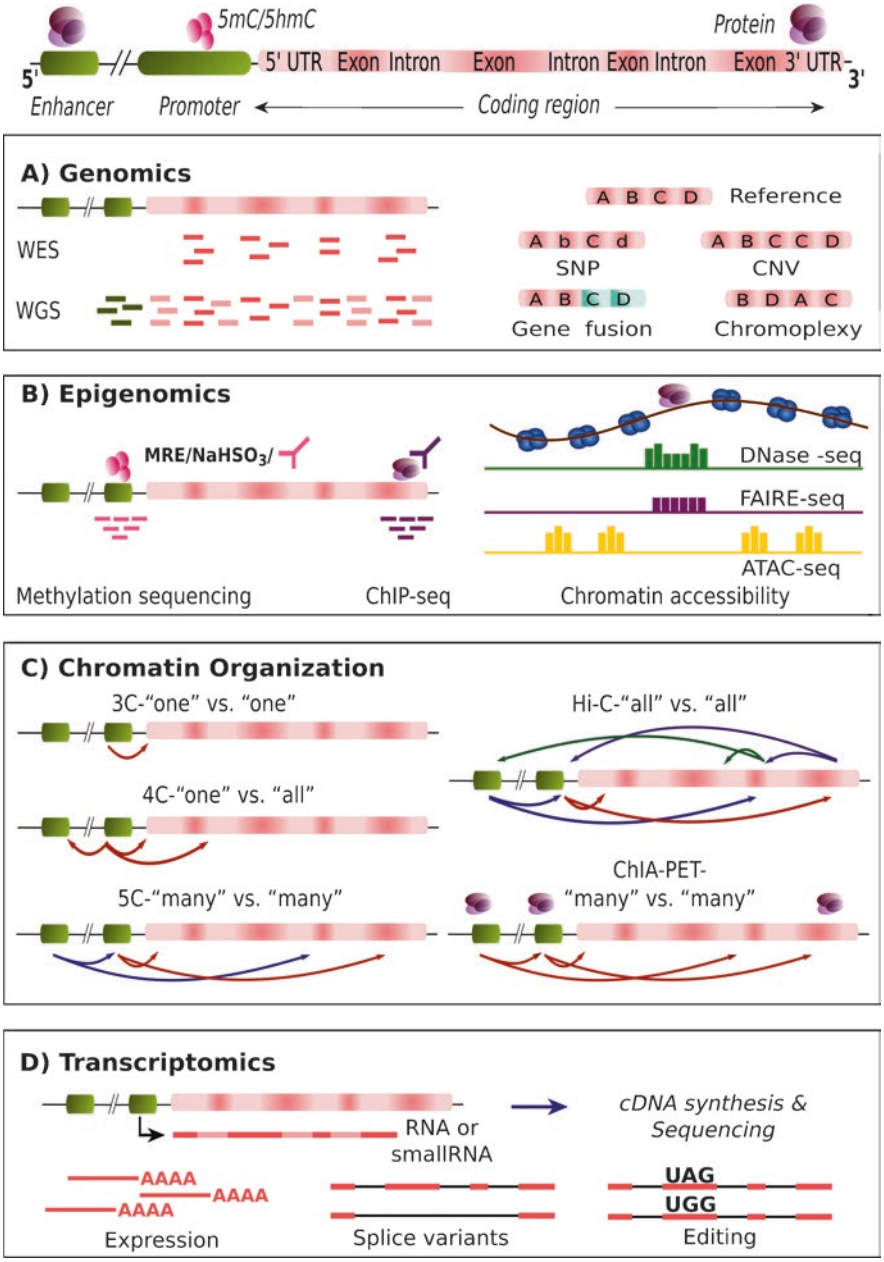

Fig. 18.3 Genomic applications for different molecular information. High-throughput sequencing allows identification and interpretation of multiple biological questions at genomic, epigenomic and transcriptomic level. (**a**) WGS and WES allow identification of SNPs, CNVs, gene fusions and other complex rearrangements like chromoplexy at the DNA level; (**b**) epigenomics include query of methylation, either by methyl-sensitive restriction enzyme (MRE), chemical modifications like sodium bisulfite conversion (NaHSO3) and immunoprecipitation, genome-wide binding (ChIP-seq) and various techniques like DNase-seq, FAIRE-seq and ATAC-seq to identify accessible regions in the genome; (**c**) crosstalks between different regions of the genome can be identified by chromosome conformation capture technologies, namely, 3C, 4C, 5C, HiC and ChIA-PET; (**d**) transcriptomic sequencing allows identification of differential gene expression, different splice variants and RNA editing events

mechanisms. The repair process randomly joins fragments together mainly using non-homologous end joining, creating many genomic rearrangements including inversions and gene fusions. This in turn creates a havoc, which can trigger the process of cancer for the fraction of cells, which can have an altered genomic situation favouring unlimited growth. It is important to emphasize that majority of the cells undergoing such catastrophe does not survive and those who does are likely purely due to chance. Such shattering can occur on one chromosome (chromothripsis) or on many chromosomes (chromoplexy). In chromothripsis, generally 1–2 chromosomes shatter into many pieces resulting in thousands of rearrangements. Chromoplexy usually involves five or more chromosomes resulting into relatively smaller number of balanced rearrangements.

Berger et al. performed WGS of prostate cancers and matched control samples and identified a characteristic chromoplexy pattern involving balanced breaking and joining which was evident in all the TMPRSS2-ERG (transmembrane protease, serine 2-erythroblast transformation-specific related gene) fusion-positive prostate cancers. This type of "closed chain" pattern also involved breakpoints among TBK1 (TANK-binding kinase 1), a NF-kB-activating kinase, TP53, MAP 2 K4 (mitogen-activated protein kinase kinase 4) and ABL1 (Abelson murine leukaemia viral oncogene homolog 1). These closed chain rearrangement breakpoints were frequently found near open chromatin regions (Berger et al. 2011).

As described in the different subsections above, all types of genomic variations can be primary drivers of cancer. These variations can act independently or in conjunction with another type of variation. Any analysis of genomic data for cancer thus should encompass a collective analysis of different data types to get a bigger and better insight. In Fig. 18.3a, we have presented a schematic view of different types of genomic variations for an overall perspective.

18.3 Epigenomic Insights into Cancer Signalling

Epigenetic changes are independent of alterations in DNA sequences, which can affect gene expression or chromatin condensation and can be heritable. Regulation of chromatin remodelling is brought about by several mechanisms involving DNA methylation, histone modifications, chromatin accessibility, chromatin-binding proteins and complex interplay among them (Jaenisch and Bird 2003; Mastroeni et al. 2011). The transcriptional state of the chromatin is a result of altered chromatin structure, either in its constituents (histone variants, promoters, repressors, enhancers or remodelling complexes) or covalent modifications of its constituents (DNA methylation or histone tail modifications). DNA methylation and post-translational histone modifications are among the most well-studied epigenetic changes that regulate cellular processes like transcription, repair and replication in highly controlled manner (Tirado-Magallanes et al. 2017; Verger and Crossley 2004). However in cancer, dysregulation of epigenetic mechanism has been reported to facilitate tumour initiation and progression (Baylin and Jones 2011; Dawson and Kouzarides 2012; Feinberg and Tycko 2004). Below we discussed different types of epigenetic changes and their impact on cancer.

18.3.1 DNA Methylation

DNA methylation occurs by transfer of methyl group from S-adenosyl methionine (SAM) to position 5 of cytosine (5mC) preferably within CpG dinucleotide (Bird 1986; Edwards et al. 2017). De novo methylation is catalysed by methyltransferases Dnmt3a and Dnmt3b while Dnmt1 maintains methylation marks (Bestor 1988; Jones and Liang 2009). 5mC is oxidized to hydroxymethyl cytosine (5hmC) by alpha glutarate-dependent oxygenases, ten-eleven translocation enzymes (TET) (Tahiliani et al. 2009). Further oxidation results in formation of 5-formyl cytosine (5fC) and 5-carboxyl methyl cytosine (5caC) readily recognized by DNA repair processes (He et al. 2011; Ito et al. 2011). While 5mC is strongly correlated with gene repression, 5hmC has been associated with elevated levels of gene expression (Chomet 1991; Doerfler et al. 1989; Gonzalez et al. 1989; Szyf 2016). 5mC and 5hmC can be detected using next-generation sequencing or microarray relying on various approaches as mentioned below.

(a) **Methyl-sensitive restriction enzyme (MRE):** Both HpaII and MspI enzymes share same recognition sequence; however HpaII digests only unmethylated DNA whereas MspI acts on DNA irrespective of its methylation status.
(b) **Covalent modifications:** Sodium bisulfite conversion specifically modifies unmethylated cytosine to uracil or 5hmC-specific glucosylation such that it becomes resistant to enzymatic cleavage.
(c) **Immunoprecipitation** of methylated or hydroxymethylated DNA using monoclonal antibody against 5mC or 5hmC.

DNA methylation affects cancer-related signalling pathways such as apoptosis, cellular proliferation, migration and cell repair (Costello and Plass 2001; Esteller 2002). Genome-wide hypomethylation (Feinberg and Vogelstein 1983; Gama-Sosa et al. 1983) in parallel with increased methylation of CpG islands (CGIs) in promoter regions (Malta et al. 2018; Weisenberger et al. 2006) is frequently observed during cancer initiation and progression. Numerous reports have shown that aberrant methylation of cancer in relevant regulatory elements modulates gene expression and consequently leads to progression of cancer. Genome-scale methylation profiling along with expression data in lung squamous carcinoma (LUSC) identified 5214 differentially methylated probes affecting expression of genes involved in ERK family, NF-kB signalling pathway and Hedgehog signalling pathway (Shi et al. 2017). Whole methylome analysis in a subtype of colorectal cancer has discovered 33 of the 160 genes involved in both canonical (WNT/β-catenin) and non-canonical (planar cell polarity (PCP) and WNT/Ca^{2+}) WNT pathway to be differentially methylated at their promoter and gene body regions (Galamb et al. 2016). Findings from another genome-wide study in gastric cancer detected promoter methylation of *KCNMA1* (potassium calcium-activated channel subfamily M alpha 1). Additionally, KCNMA1 was shown to be negatively associated with survival, induced cell apoptosis in vitro and suppressed xenograft tumour growth in subcutaneous mouse models by targeting PTK2 (Ma et al. 2017). *FOXF2* (forkhead

box F2) is another transcription factor within the WNT signalling pathway, found to be preferentially methylated in gastric cancer. Ectopic expression of FOXF2 inhibits Wnt signalling by inducing β-catenin protein ubiquitination and degradation, thereby suppressing carcinogenesis (Higashimori et al. 2018). Thus significant advances in sequencing technologies have led to identification of genes with differential DNA methylation status implicated in cancer-associated pathways. These findings provide novel opportunities for development of methylation-based biomarkers in cancer diagnostics and therapeutics.

18.3.2 Histone Modification

Histone proteins, which package and order DNA into nucleosomes, can undergo alterations at their N- and C-terminal tails. Covalent modifications like histone tail methylation, acetylation, ubiquitination, sumoylation and phosphorylation determine the chromatin state (active or inactive). Thus, these proteins play a crucial role in regulating the gene expression profile (expressed or repressed), and aberrant histone profiles can result in malignant cellular transformation. Till recently technical limitations have enabled epigenetic profiling of only few hundred loci implicated in tumour progression and promotion. Nevertheless, cancer being a heterogeneous disease requires whole genome profiling of epigenetic landscape at single base-level resolution (Chrun et al. 2017; Vidal et al. 2017). Integration of genome-scale sequencing technologies with traditional techniques to study methylation and histone modifications has paved way for comprehensive understanding of epigenetic mechanisms and their role in cancer (Stirzaker et al. 2014).

Chromatin immunoprecipitation followed by sequencing (ChIP-seq) allows identification of DNA-protein interactions in vivo on a genome-wide scale, revealing transcriptional regulatory network. It is used to map the occupancy of transcription factors or other chromatin proteins like histones, across the whole genome. It is based on crosslinking the nuclei to fix protein-DNA interactions, followed by fragmentation and enrichment. This is followed by reverse crosslinking and enriched DNA is purified and put through high-throughput sequencing. This is a powerful technique in mapping the regulatory profile for various factors, especially where the context of specific cell types is important, like that of cancer.

Chromatin framework determines accessibility of DNA to transcription factors and chromatin remodellers which mediate gene expression (Quina et al. 2006). Several histone-modifying enzymes (histone demethylases, deacetylases, methyltransferases, ubiquitinating enzymes, etc.) are responsible for the dynamic addition or removal of the various reversible post-translational modifications to histone tails (Kouzarides 2007b; Marks et al. 2001; Pedersen and Helin 2010). Epigenetic deregulation in histone-modifying complexes and histone marks play a major role in development and progression of diseases, especially cancers (Sharma et al. 2010), via abnormal gene transcription and DNA repair. Histone modifications can be diverse, depending on the histone and the amino acid residue undergoing the modification (lysine residues accepting mono-, di- or tri-methylation and arginine

residues being mono- or di-methylated). Different combinations of histone modifications ("histone code") result in different biological outcomes.

Lysine acetylation is one of the most widely seen histone modifications in eukaryotes. The addition is brought about by histone acetyltransferases (HATs) (Loidl 1994) and histone deacetylases (HDACs) (Brownell and Allis 1995) responsible for reversing this modification. Together the HATs and HDACs maintain the dynamic equilibrium of lysine acetylation in vivo. The various molecules involved in these multi-protein complexes ensure proper substrate specificity and locus targeting. Lysine acetylation allows conformational changes (Luger and Richmond 1998; Norton et al. 1989) in chromatin, "opening" it for different regulating factors. Hyper acetylation of histones is considered as a hallmark of transcriptionally active chromatin.

Histone methylation occurs on side-chain nitrogen of histone (commonly H3 and H4) lysine and arginine residues. Protein arginine methyltransferases (PRMTs) catalyse the arginine methylation. Type I PRMTs catalyse mono- and asymmetric arginine di-methylation, whereas type II enzymes catalyse mono- and symmetric arginine di-methylation (Gary and Clarke 1998). Similarly, lysine methylation also happens as mono-, di- or tri-methylated forms. Some of the lysine residues undergoing methylation are also substrates for acetylation. Lysine residue methylation enzymes can be lysine-specific SET domain containing histone methyltransferases (HMTs) and the non-SET containing HMTs. Lysine methylation has widespread consequences. The lysine residue undergoing methylation can determine the transcriptional status of the gene (Sims et al. 2003). Heterochromatin (closed chromatin) is marked by H3K9, H4K20 and H3K27 methylation, whereas euchromatin (open chromatin) is marked by H3K4, H3K36 and H3K79 methylation. Bivalency or "stemness" in embryonic stem cells is showcased by a tightly regulated balance between H3K4 and H3K27 methylations, to maintain pluripotency (Bernstein et al. 2006). Lysine methylations are reversed by lysine demethylase (LSD1; demethylate mono- and di-methyl K4 on H3) (Y. Shi et al. 2004), jumonji-domain-containing demethylase (JHDM1A; demethylates mono- and di-methyl K36 on H3) (Tsukada et al. 2006) and JMJD2/KDM4 demethylases (tri-methyl demethylase family) (Whetstine et al. 2006) and arginine demethylase (Chang et al. 2007).

Histone phosphorylation alters chromatin structure and facilitates transcription factor interaction with chromatin elements. Histones H1, H2A, H2B, H3 and H4 are susceptible to phosphorylation at serine or threonine residue. Various kinases are needed for histone phosphorylation on distinct residues. H2B S14 phosphorylation plays a role in apoptosis and catalysed by Mst1 (mammalian sterile 20-like kinase) (Ahn et al. 2005). Aurora kinases phosphorylate H3S10 and H3S28, inducing transcriptional activity during mitosis (Nowak and Corces 2004). In contrast, protein phosphatase 2 (PP2A) dephosphorylates H3S10, inhibiting transcriptional activity (Nowak and Corces 2004).

In vivo, histones H1, H2A, H2B and H3 can be ubiquitinated at lysine residues, where modification of H2A and H2B is more common (Zhang 2003). Although histone ubiquitination is generally considered as mark of "open" chromatin, they have been linked to both gene activation and suppression (Zhang 2003). Ubiquitinated

histones are reported to recruit additional regulatory molecules, for successive histone modifications (Briggs et al. 2002). As histone ubiquitination has control of successive methylation and acetylation, it can lead to both transcriptional activation and suppression.

Histone variants are not common and are generally seen in H2A and H3 family of histones, besides the linker histone H1. They can have temporal and spatial specific expression, and they need histone chaperones to be incorporated. They have been reported to be of prognostic value in cancers (Hua et al. 2008; Sporn and Jung 2012; Sporn et al. 2009) and also involved in cancer progression (Dardenne et al. 2012; Kapoor et al. 2010; Novikov et al. 2011; Schwartzentruber et al. 2012; Svotelis et al. 2010; Wu et al. 2012).

As epigenetic research is gaining strong hold, there have been multiple reports of aberrant profiles, resulting in abnormal silencing of tumour suppressors or activation of oncogenes (Feinberg and Tycko 2004; Jones and Baylin 2002), leading to deregulated proliferation, invasion, metastatic progression and therapy resistance (Gronbaek et al. 2007; Gupta and Massague 2006). As epigenetic modifications are crucial in maintaining the balance between pluripotency and differentiation in normal stem cell populations, disruptions in usual profile may shift the equilibrium towards oncogenesis (Feinberg et al. 2006; Niwa 2007). Studies revealed primary non-small cell lung cancer (NSCLC) recurrence was associated with low levels of H3K9me3, H4K16ac and H3K9ac (Song et al. 2012), whereas the ones with large cell or squamous cell carcinoma (who had higher survival rates) demonstrated high levels of H3K4me2, and stage I cases with adenocarcinomas had low H3K9ac levels (Barlesi et al. 2007). Adenocarcinoma patients with poor survival have been shown to have lower levels of H3K4me2 and H3K18ac (Seligson et al. 2009). Increased H3K27 methylation and EZH2 (its specific methyltransferase) were associated with prostate cancer progression and metastasis (Ellinger et al. 2012; Varambally et al. 2002) and breast cancer (Kleer et al. 2003; Pietersen et al. 2008). Genome-wide lower levels of H3K9ac, H3K18ac, H3K4me1, H3K4me2, H3K9me2, H3K9me3, H4K5ac, H4K8ac, H4K16ac, H4K20me3 and H4R3me2 in tumour tissues were associated with poor prognosis in a number of cancers (Chervona and Costa, 2012).

18.3.3 Chromatin Accessibility

DNA is packed in the nucleus as nucleosomes, a histone octamer core bound by DNA (Kornberg 1974; Luger et al. 1997; Richmond and Davey 2003). The core has four basic histones (Luger et al. 1997), which can be post-translationally modified by covalent alterations (Bannister and Kouzarides 2011; Kouzarides 2007a) or replaced by histone variants (Hake and Allis 2006; Henikoff and Ahmad 2005; Szenker et al. 2011). This nucleosomal organization plays a significant role in recruitment of regulatory proteins, and regulating availability of DNA binding sites to general transcriptional machinery and transcription factors, thus governing processes like transcription, DNA replication, recombination and repair (Radman-Livaja and Rando 2010). The accessible (open for recruitment) chromatin regions

are hence considered as primary regulatory elements and are characterized by nuclease hypersensitivity. Genome-wide profiling of chromatin accessibility is an approach to identify crucial epigenetic alterations that contribute to development, signalling and disease development, where ENCODE (Consortium 2012) serves as a registry of functional elements of the genome. DNase-Seq, FAIRE-seq and ATAC-seq are direct chromatin accessibility assays, where the open chromatin is determined by hypersensitivity to nucleases.

DNase I hypersensitivity is fundamental to active cis-regulatory DNA regions including promoters, enhancers, insulators and locus control regions. DNase hypersensitivity sites (DHS) are bordered by nucleosomes, which may gain histone modifications that mimic functional role of the adjacent regulatory DNA, such as association with histone H3 lysine 4 trimethylation (H3K4me3) with promoters (Heintzman et al. 2007). Next-generation sequencing era allowed genome-wide identification of DHS. It has been used by the ENCODE consortium to reveal cell-specific chromatin accessibility and its relation to gene expression in various cell lines (Boyle et al. 2008; John et al. 2011; Thurman et al. 2012). DNase I has been modified to reflect rotational positioning of individual nucleosomes (Winter et al. 2013) based on the preference of DNase I to cut within DNA minor groove around nucleosomes (Boyle et al. 2008; Cousins et al. 2004; Noll 1974). Besides, DNase I cleavage intensity depends on bound sequence-specific regulatory proteins within DHS and creates digital genomic footprint (DGF; or DNase I footprinting). DGF allows study of transcription factor occupancy at nucleotide resolution (Hesselberth et al. 2009). However, DNase I is reported to have cleavage bias (Boyle et al. 2008; Cousins et al. 2004; Hesselberth et al. 2009; Noll 1974; Sung et al. 2014; Zhang and Pugh 2011), hence questioning its usage as a reliable transcription factor footprinting assay.

FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements)-seq is another way of isolating nucleosome-depleted genomic regions. Its widespread use is restricted by high background in the output data. It is based on phenol-chloroform separation of nucleosome-bound and "open" genomic areas in interphase and aqueous phase, respectively. This also involves a prior crosslinking step with formaldehyde to capture the DNA-protein interactions and successive sonication of the chromatin. FAIRE signatures have been reported to be negatively associated with nucleosome occupancy and positively with cell-specific open chromatin (Buck et al. 2014; Gaulton et al. 2010; Giresi et al. 2007; Song et al. 2011). It has been reported to be associated with specific chromatin states with known sequence variants of disease susceptibility (Gaulton et al. 2010) or allele-specific signature (Yang et al. 2013). This has an advantage that the nucleosome deprived is not degraded, and it allows identification of additional distal regulatory regions when compared to DNase-Seq, as well as having no sequence-specific bias. However, it has low efficiency when it comes to highly expressed gene promoters (Song et al. 2011). But the major drawback so far is the low signal-to-noise ratio, making interpretation difficult.

ATAC (assay for transposase-accessible chromatin)-seq is the most recent of the chromatin accessibility assays. It depends on the hyperactive Tn5 (Adey et al. 2010;

Goryshin and Reznikoff 1998) to cut the DNA and integrate into open regulatory regions in vivo (Buenrostro et al. 2013). When the cell nuclei are tagged in vitro with the adapters and purified Tn5, a majority of adapters are incorporated into regions of open chromatin due to steric hindrance, which is taken onto high-throughput sequencing. It has been used to profile open chromatin, nucleosome positioning (limited to regions in close proximity to open sites) and transcription factor footprints (Buenrostro et al. 2013). It has similar efficiency as that of DNase-Seq data, in terms of sensitivity and specificity, but the number of cells required is three to five order of magnitudes more.

All the above chromatin accessibility assays are inherently limited by their principle of identifying open chromatin depending on DNA fragmentation. Annotation of the open chromatin regions is highly dependent on availability of transcription factor binding motifs and relevant information along with spatial and temporal interaction of DNA with transcription factors (He et al. 2014; Sung et al. 2014).

The combination of histone modification and nucleosome position profiling plays a crucial role in gene regulation, governing development and differentiation. Nucleosome remodelling and deacetylase (NuRD) corepressor complex has been reported to play a central role in aberrant gene silencing in leukaemia via the oncogenic transcription factor, PML-RARa (Morey et al. 2008). ATP-dependent chromatin remodelling complex, SWI-SNF complex modifications, is also associated with cancer development (Reisman et al. 2009). H2A.Z, a histone variant, has also been implicated in tumorigenesis. It has been shown to be upregulated in several types of cancer and has been linked with cell cycle progression (Svotelis et al. 2009), whereas its lower levels have been associated with tumour progression via modulating chromatin remodelling resulting in formation of closed chromatin and hypermethylation of tumour suppressor gene promoters (Witcher and Emerson 2009).

As evidenced from the above section, similar to the genomic variations, epigenomic variations can also play havoc with the dynamic equilibrium of cellular homeostasis. By the very nature, epigenomic changes are more dynamic than the genomic variations and thus require tightly controlled experimental strategies to decipher a molecular signature underlying a phenotypic change. In Fig. 18.3b, different subtypes of epigenetic changes are depicted schematically.

18.4 Long-Range Control of Transcription and Its Role in Cancer Signalling

The three-dimensional organization of the genome into predefined compartment regulates various cellular processes like replication, transcriptional regulation, cellular differentiation, etc., by bringing together distal regulatory elements and genes in direct physical association via chromatin interactions (Misteli 2007; Kosak and Groudine 2004; Cremer and Cremer 2001). There are numerous techniques to measure spatial organization of chromatin. Conventional methods include light and electronic microscopic imaging (Cremer et al. 2006; Bolzer et al. 2005; Cremer et al. 1982) and DNA fluorescence in situ hybridization (FISH) techniques (Simonis

and de Laat 2008). On the other hand, chromosome conformation capture (3C) methodologies and derivatives rely on molecular biology-based approach and allow capture of both global and local, long-range interactions.

3C technique and its modifications are nuclear ligation-based approaches to detect chromatin interactions in their native state. The initial steps involve fixing and formaldehyde crosslinking of the nucleus, enzymatic chromatin digestion and proximity ligation followed by detection of chimeric products. 3C, first developed in 2002, detects interaction frequency between known genomic regions ranging from ten to a few hundred kilobase pairs and requires prior knowledge of genomic locations of the elements to be analysed (Hagege et al. 2007; Dekker et al. 2002). As such the technique is confined to analysis of locus that is relatively closer on linear DNA and does not allow parallel investigation of a large number of loci and also unknown regions. Recent advances in 3C technology, owing to next-generation sequencing (NGS) and microarray, have made it possible to examine both locus-specific and genome-wide long-range chromatin interactions at higher resolution.

In circular chromosome conformation capture (4C) technology, the genome is screened for interactions against a known genomic region ("bait"). Genomic fragments ligated to the "bait" fragment are amplified and detected either by inverse PCR, sequencing or microarray (Splinter et al. 2011; Zhao et al. 2006; Simonis et al. 2006). Chromosome conformation capture on chip (5C) allows simultaneous mapping of interactions between multiple selected genomic fragments using ligation-mediated amplification (LMA). This allows simultaneous amplification of many 3C junctions. The resulting PCR amplicons are detected by either microarray analysis or deep sequencing (Dostie et al. 2006). High-throughput chromosome conformation capture (Hi-C) allows inspection of genome-wide, long-range chromatin interaction through massively parallel sequencing in an unbiased manner (van Berkum et al. 2010; Lieberman-Aiden et al. 2009). Hi-C has revealed organization of genome into topologically associated domains (TADs) or contact domains, which are several hundred kilobases to one megabase in length. These boundaries are conserved across many cell types but the chromatin profile within a TAD is dynamic (Nora et al. 2012; Dixon et al. 2012). Capture Hi-C is a modification of Hi-C protocol whereby cis-interactions across multiple selected regions can be captured at high-resolution using site-specific hybridization probes (Hughes et al. 2014). Chromatin organization often requires architectural protein like CCCTC-binding factor (CTCF) and cohesin for its maintenance (Hansen et al. 2017, Ong and Corces, 2014). CTCF along with cohesin induces chromatin looping between distant genes, thereby influencing expression within a TAD boundary (Vietri Rudan et al. 2015; Guo et al. 2015). Chromatin interaction analysis by paired-end tag sequencing (ChIA-PET) combines immunoprecipitation and Hi-C to identify protein-mediated chromatin interaction at genome-wide level (Li et al. 2010; Fullwood et al. 2010; Fullwood et al. 2009). Although revolutionary, these techniques are meant to study the existence of chromatin interaction and do not comment on the functionality of the chromatin loop. In order to assess the biological significance of an interaction identified by 3C-based technologies, additional genetic experiments are required. Since its inception in early 2000, the 3C techniques have been implemented both in

healthy and disease scenario, including cancer (Jia et al. 2017; Achinger-Kawecka and Clark 2017; Babu and Fullwood 2015). Chromatin organization regulates various cancer-relevant cellular processes; consequently any abnormalities in chromatin architecture may facilitate tumour progression. Accumulation of genomic variation like segmental duplications, base substitutions or translocation can facilitate aberrant chromatin interaction (Taberlay et al. 2016; Schuster-Bockler and Lehner 2012; Fudenberg et al. 2011; Roix et al. 2003). Disruption of said interactions may influence transcriptional and epigenetic regulation by mediating spatial co-localization between target gene and active/repressive regulatory elements (Rickman et al. 2012; Elemento et al. 2012; Tiwari et al. 2008). This may affect expression of cancer-related genes, thus triggering altered physiological behaviour and malignancy. The following section will focus on recent development in high-throughput chromosome conformation capture techniques in understanding long-range control of cancer signalling.

18.4.1 Interactions Between Cancer Risk-Associated Alterations and Candidate Genes

Variation in genomic sequence may result in altered chromosome conformation. 3C derivatives are routinely used to identify long-range interactions among risk-associated genomic variations like copy number variants (CNVs) or single nucleotide polymorphisms (SNPs) with candidate genes in different cancers. Corroborative evidence confirms that *FOXA1* (forkhead box protein A1) favours reference (T) allele over the alternate (G) allele of risk-associated SNP, rs4442975, in breast cancer (Dryden et al. 2014). FOXA1 has been shown to influence ER estrogen receptor-α -interactions with chromatin and regulate endocrine response in breast cancer cells (Hurtado et al. 2011). Chromatin interactions containing risk SNPs in testicular germ cell tumour (TGTCs) have identified target genes involved in developmental transcriptional regulation, microtubule or chromosomal assembly, defective KIT-MAPK signalling (Litchfield et al. 2017). Additionally, there are numerous studies that explore chromatin loops involving cancer risk-associated genomic alterations (Table 18.1). Of note, the interaction between MYC proto-oncogene and risk-associated locus 8q24.21 has been observed in multiple cancers, for example, breast cancer, colorectal cancer and prostate cancer. Together, these findings suggest a link between genomic alterations and disruption of local chromatin interactions. In future, these interactions can be further harnessed for their therapeutic potentials.

18.4.2 Chromatin Interaction and Possible Disruption in Cancer

As previously mentioned, TAD boundaries are conserved across cell types. However, cancer exhibits additional TAD boundaries resulting in smaller TADs; like in prostate cancer a common deletion at 17p13.1 within TP53 tumour suppressor locus

Table 18.1 Chromatin loops involving cancer risk-associated genomic alterations

S. No	Technology	Cancer type	Loci	Gene symbol	Ref (PMIDs)
1	Hi-C	Breast cancer	2q35	*IGFBP5, DIRC3*	25122612
			8q24.21 9q31.2	*MYC, CCDC26, NSMCE2, PVT1, KLF4, RP11-363D24.1*	
2		Hepatocellular carcinoma	7q21.13	*CDK14*	29246937
3		Glioma	2q33.3	*IDH1*	29460007
			8q24.21	*MYC*	
			20q13.33	*STMN3*	
4		Prostate cancer	7p14.3	*SPOP*	28663546
5		Prostate cancer	15q21.3	*AP-1*	22496589
6	Capture Hi-C	Colorectal cancer	8q24.21	*MYC*	25695508
			3q26.2	*MECOM*	
7	4C-seq, 3C-seq	Prostate cancer	8q24	*POU5F1B, MYC, CD96*	26934861
					25149474
8	3C-seq	Prostate cancer	2p11.2	*CAPG*	26979803
			2p24.1	*C2orf43*	
			6q22.1	*RFX6*	
			1q32.1	*NFASC*	
			8q24.1	*MYC*	
			10q11.23	*AGAP7P*	

results in division of a single TAD into two distinct smaller TADs (Taberlay et al. 2016; Achinger-Kawecka et al. 2016). Disruption and formation of new boundaries can initiate novel interactions by bringing together distal cis- or trans-acting regulatory elements and genes in close spatial proximity in cancerous cells. For instance, in acute myeloid leukaemia (AML), inversion at chromosome 3 rearranges TAD boundary to allow direct physical association between distal GATA2 and EV1 (enhancer and stem cell regulator) genes (Groschel et al. 2014). In breast cancer cells, RUNX1 (runt-related transcription factor 1) is enriched at TAD boundaries and regulates expression of genes involved in chromatin structure and extracellular matrix formation like FN1 (fibronectin 1) and FBN2 (fibrillin 2) by mediating long-range local interactions (Barutcu et al. 2016). Another study reported interaction of breast cancer-related genes IGFBP3 (insulin-like growth factor binding protein 3) and EGFR (epidermal growth factor receptor) and associated interaction only in breast cancer cells (MCF-7, MDA-MB-231) and not in normal breast cell (HMEC) (Zeitz et al. 2013). These findings strongly implicate disruption of long-range interaction and associated deregulation of transcription in cancer.

Genome organization weaves together genomic, transcriptomic (discussed below) and epigenomic alterations, hence contributing to gene regulation in cancerous cells. Findings from numerous studies have contributed towards understanding of complex architecture of cancer genome. Although this enhances our knowledge of basic biology of cancer, the data generated on high-throughput 3C platform

represents averaged genome topology of a cell population. Moreover, 3C derivatives neither match the resolution offered by electron microscopy nor capture the dynamic interactions in single cells as DNA-FISH. Whereas for translational and clinical purposes, it is essential to characterize chromatin interaction in patient samples at high resolution. Recently developed genome engineering technique and single-cell Hi-C can help in exploring the changes in genome architecture during various stages of cancer progression in clinical samples. Insights gained from such efforts will encourage development of chromatin organization-based therapeutic approaches for better management of cancer. In Fig. 18.3c, different aspects of long-range control of transcription are depicted.

18.5 Transcriptomic Insights into Cancer Signalling

DNA across all cells of an organism is largely identical whereas the actively transcribed RNA is highly dynamic and largely responsible for the diversity and differential regulation across varied cell types. Northern blot is a highly specific and the first technique developed to detect RNA (Alwine et al. 1977), but low sensitivity limits its applications. The discovery of quantitative RT-PCR (qRT-PCR) made the detection more sensitive but expression of only few genes could be queried by both these techniques. Discovery of microarray allowed the detection of multiple genes in parallel (Schena et al. 1995) but can't be used for detecting novel transcripts. Quantifying the transcriptome including detection of novel genes, alternative splice sites as well as variations was made possible by the advent of RNA sequencing (Lister et al. 2008; Mortazavi et al. 2008; Nagalakshmi et al. 2008). Transcriptomic analysis in a disease-like cancer allows us to study different stages of progression to understand molecular mechanism at early and advanced stages of cancer (Smith et al. 2005; Thomas et al. 2013). Another unique feature is stratification of cancers where distinct subtypes can be identified for better treatment and management (Bertucci et al. 2005; Chandran et al. 2015; Liang et al. 2005; Yan et al. 2012). Here we will discuss the impact of microarray and next-generation sequencing technologies like RNA-seq and small RNA-seq and their impact in better understanding of cancers.

18.5.1 Gene (mRNA) Expression

Dhanashekaran et al. did gene expression profiling for more than 50 normal and neoplastic prostate samples to molecularly distinguish between normal, benign prostatic hyperplasia and localized and metastatic prostate cancer (Dhanasekaran et al. 2001). Interestingly, this paper along with others has identified hepsin to be upregulated in cancer using gene expression profiling (LaTulippe et al. 2002; Luo et al. 2001; Magee et al. 2001). One of the mechanisms by which hepsin promotes prostate cancer progression and metastasis (Klezovitch et al. 2004) is by specifically cleaving an extracellular matrix molecule, laminin-332, and enhancing motility of

prostate cancer cells, which is inhibited by a hepsin inhibitor (Kunitz domain-1) (Tripathi et al. 2008). RNA sequencing of 20 primary neuroblastomas with and without MYCN amplification, followed by pathway analysis, revealed upregulated genes from mTOR (mammalian target of rapamycin) pathway. MYCN-driven neuroblastoma mice model also showed upregulation of the mTOR pathway (Schramm et al. 2013). Interestingly, inhibiting mTOR resulted in downregulation of MYCN and inhibition of neuroblastoma growth in vitro and in vivo (Johnsen et al. 2008). Combination of next-generation sequencing and microarray in breast cancer stem cells identified upregulated genes, which drive the PI3K pathway (Hardt et al. 2012). Microarray data from colon adenocarcinoma cell line identified MEGF6 (multiple epidermal growth factor-like domains protein 6) to be upregulated which was also validated from TCGA-COAD (The Cancer Genome Atlas-Colon adenocarcinoma) cohort and correlated with poor survival MEGF6 promoted cell proliferation and inhibited apoptosis. Additionally MEGF6 promoted metastasis both in vitro and in vivo. MEGF6 induced SNAI2 (snail family transcriptional repressor 2) and triggered epithelial-to-mesenchymal transition (EMT) via TGF/SMAD signalling pathway (Hu et al. 2018).

18.5.2 MicroRNA Expression

Primarily driven by large-scale networked research programmes, we now know that majority of the transcribed genome remains non-coding. MicroRNAs are the major class of non-coding RNA molecules with a proven role in cancer. Below we will discuss major findings from this area of research.

One of the most well-known onco-miRNA, miRNA-21, was identified in GBM by microarray in primary glioma samples and knockdown of miRNA-21 triggered caspase activation and subsequent apoptosis (Chan et al. 2005). Subsequent studies characterized miRNA-21 as a bona fide onco-miRNA in multiple cancers (Gabriely et al. 2008; Li et al. 2009; Xiong et al. 2012). miRNA-21 targets multiple tumour suppressors, like PDCD4 (programmed cell death protein 4), PTEN (phosphatase and tensin homolog), RECK (reversion-inducing cysteine-rich protein with kazal motifs), TIMP3 (metalloproteinase inhibitor 3), etc., leading to resistance of apoptosis, increased proliferation and invasion (Asangani et al. 2008; Gabriely et al. 2008; Lu et al. 2008; Zhang et al. 2012). Let-7 was identified as a tumour suppressor miRNA in liver and lung cancers by microarray and targets multiple oncogenes including K-RAS, C-MYC, HMGA2 and cell cycle factors, like cyclins D1 and D2 (Johnson et al. 2005; Roush and Slack 2008). Microarray analysis of nasopharyngeal carcinoma (NPC) cells and immortalized nasopharyngeal epithelium cells identified 27 upregulated and 18 downregulated miRNAs (Yi et al. 2012). Interestingly, miRNA-663 was identified to be a tumour suppressor in gastric cancer (Pan et al. 2010) but was found to be upregulated in NPC cells. p21 (cyclin-dependent kinase inhibitor 1A) was identified as a target for miR-663. p53 transcriptionally regulates p21, inducing p53-mediated cell cycle arrest at the G1 phase, which is abrogated in p21-deficient cells (Waldman et al. 1995). So, downregulation

of p21 by miR-663 promoted G1/S transition and proliferation in NPC cells (Yi et al. 2012). Another miRNA-based microarray study identified miRNA-486 as the most downregulated miRNA in stage 1 adenocarcinoma patients compared to adjacent normal tissues (Peng et al. 2013). miRNA-486 targets multiple genes from insulin growth factor (IGF) signalling including IGF, IGF1 receptor (IGF1R) and phosphoinositide-3-kinase, regulatory subunit 1 (PIK3R1). In vitro miRNA-486 reduced growth and migration and induced apoptosis whereas overexpression of miRNA-486 in adenocarcinoma cell lines resulted in tumour volume reduction in vivo (Peng et al. 2013). Microarray analysis of hepatocellular carcinoma (HCC) samples identified 35 differentially expressed miRNAs, out of which miRNA-122a, an abundantly expressed miRNA in liver, was found to be downregulated in HCC (Gramantieri et al. 2007). Cyclin G1 (CCNG1) was identified as a target of miRNA-122a. The follow-up study showed that miR-122a, via CCNG1, influences p53 stability leading to reduced invasion and increased sensitivity to doxorubicin in HCC (Fornari et al. 2009).

Recently, clustered miRNAs in cancer have gained a lot of importance (Laddha et al. 2013; Olive et al. 2010; Song et al. 2016). Evidence suggest that miRNA cluster targets same or similar genes in the same pathway (Bartel 2004; Grun et al. 2005; Kim and Nam 2006; Yu et al. 2006) possibly leading to coordinated functions of large miRNA clusters (Laddha et al. 2013). Microarray and NGS analysis from different cancers, including GBM from TCGA data portal, revealed miR-379/miR-656 cluster to be downregulated in multiple cancers (Laddha et al. 2013). Among these miRNAs, miRNA-134 was predicted to target the maximum number of genes in the "glioma pathway". Luciferase validation identified epidermal growth factor receptor (EGFR) and a serine/threonine kinase, RAF1, as the target of this miRNA in GBM (Nayak et al. 2018).

18.5.3 RNA Splicing

NGS not only allows detection of differentially expressed genes between cancers and normal but also post-transcriptional modifications like RNA splicing and RNA editing.

Alternative splicing allows a gene to produce multiple splice variants and protein isoforms with different functions and thus its dysregulation can have a huge impact in a disease like cancer (Climente-Gonzalez et al. 2017; Oltean and Bates 2014). Neuroendocrine prostate cancer (NEPC) is an aggressive subtype of prostate cancer, which does not respond to androgen receptor pathway inhibition (ARPI). Whole transcriptome sequencing identified a NEPC-specific splicing signature, majority of which is controlled by serine/arginine repetitive matrix 4 (SRRM4) (Y. Li et al. 2017). In vitro and in vivo evidence confirmed RE1 silencing transcription factor (REST), a master neural differentiation regulator, to be a splicing target of SRRM4 and increases the expression of dominant negative REST4 splice variant, resulting in a neuroendocrine transdifferentiation (Collet-Cassart et al. 1989; Li et al. 2017). NEPC shows resistance to cell death by APRI, radiation and chemotherapy

(Collet-Cassart et al. 1989). Interestingly, Bax-interacting factor 1 (*Bif-1*) is differentially spliced in NEPC (Y. Li et al. 2017). Bif-1 interacts with UV radiation resistance associated (UVRAG), Beclin-1, to regulate mitochondrial dynamics and tumorigenesis (Takahashi et al. 2007). Bif-1 via its N-BAR (Bin-Amphiphysin-Rvs) interacts with Bcl-2-associated X protein (Bax) to activate it and promote cytochrome-c release (Cuddeback et al. 2001). *Bif-1* has three splice variants *Bif-1a*, *Bif-1b* and *Bif-1c*. Bif-1a is ubiquitous whereas Bif-1b and -1c are brain specific (Modregger et al. 2003). SRRM4-mediated splicing results in conversion of pro-apoptotic Bif-1a into anti-apoptotic Bif-1b and Bif-1c, which has inclusion of micro-exons within the N-BAR domain and might result in loss of interaction with Bax (Gan et al. 2018; Lee et al. 2018; Wang et al. 2014). Hyper activation of NOTCH pathway is common in human lung adenocarcinomas and correlates with poor prognosis (Dang et al. 2000; Westhoff et al. 2009). NUMB (protein numb homolog) inhibits NOTCH pathway, promoting ubiquitination and proteosomal degradation (McGill and McGlade 2003). Splicing-sensitive microarrays identified NUMB as one of the alternatively spliced targets upon knockdown of RNA binding motif protein 5 (RBM5), RBM6 and RBM10. Interestingly, high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP) identified a peak for RBM10 in the 3′ splice site preceding NUMB exon 9 and overexpression of RBM10 led to exon 9 skipping whereas overexpression of RBM6 led to exon 9 inclusion. Inclusion of exon 9 in NUMB has been shown to be frequent in lung cancers; this alternative splicing results in reduced level of NUMB protein leading to activation of NOTCH signalling (Misquitta-Ali et al. 2011). Intriguingly, lung adenocarcinoma cell line A549 expressed a mutant RBM10 having reduced activity for exon 9 skipping, resulting in increased NOTCH signalling (Bechara et al. 2013). Transcriptome sequencing in 20 breast cancer samples including ERBB2-amplified (Erb-B2 receptor tyrosine kinase 2), ESR1-amplified (estrogen receptor 1), and triple-negative tumours and matched breast tissue controls revealed intronic and exonic overexpression of ERBB2 and its neighbouring genes in ERBB2-amplified tumours. Identification of alternatively spliced tumour-specific transcripts is critical because it might render drugs like Herceptin, which targets the full-length ERBB2, less effective (Forootan et al. 2016). Additionally, tumour showing amplified ESR1 or with intronic overexpression is retained in the cytoplasm impairing its transcription factor ability, which will again lead to impaired drug therapy response (Forootan et al. 2016).

18.5.4 RNA and miRNA Editing

RNA editing covers the broad array of changes in nucleotide sequences within mature mRNAs and can be categorized into insertions/deletion and substitution. Insertion/deletion was first identified in kinetoplast mitochondrion of *T. brucei* (Benne et al. 1986). In animals, the substitution type of RNA editing mainly involves A-to-I (mediated by adenosine deaminase acting on RNA, ADAR) and C-to-U

(mediated by apolipoprotein B mRNA editing enzyme catalytic subunit 1) changes which are enzyme mediated (canonical editing). Recently, there are also reports of non-canonical editing events for which the mechanisms are not yet known and broadly classified as RNA-DNA differences (RDD) (Li et al. 2011). Both hypoediting and hyperediting have been reported in cancers (L. Han et al. 2015; Kang et al. 2015; Paz et al. 2007). RNA-seq of HCC tumours and their adjacent non-tumour tissues identified a non-synonymous A-to-I editing (serine to glycine change) in antizyme inhibitor 1 (AZIN1). The editing induced a cytoplasmic-to-nuclear translocation, increased affinity to antienzyme and promotion of cell proliferation by degrading ODC (ornithine decarboxylase) and CCND1 (Chen et al. 2013). Another transcriptomics study in HCC patients and matched control liver tissues detected 485,684 edits in 18 samples, resulting in identification of 292 differential edits (Kang et al. 2015). Interestingly, the edited genes were also differentially expressed and three out of the four top differentially expressed genes, caspase 2 (CASP2), MAF bZIP transcription factor K (MAFK) and unc-51 like autophagy activating kinase 2 (ULK2) (Dawar et al. 2016; John Clotaire et al. 2016; Okita et al. 2017). In an effort to identify *driver* editing events, RNA-seq for 17 different cancers from TCGA was analysed and 35 non-synonymous RNA editing sites were identified, out of which eight events were identified in more than one cancer type. Edited version of AZIN1, GRIA2 (glutamate ionotropic receptor AMPA type subunit 2) and COG3 (component of oligomeric Golgi complex 3) was shown to affect the sensitivity of IGF1R inhibitors and MEK inhibitors (Han et al. 2015). Intriguingly, GRIA2 (or GluR-B) are excitatory neurotransmitter receptors and RNA editing is an absolute requirement for their proper functioning in normal brain. The editing event, mediated by ADAR2, leads to a glutamine to arginine (Q/R) change making the channel impermeable to Ca^{2+} (Higuchi et al. 1993; Seeburg and Hartner 2003). Absence of editing at this position leads to postnatal lethality in mice due to excess influx of Ca^{2+}, which can be reversed by restoring the edited version of GRIA2 (Higuchi et al. 2000). Additionally, hypoediting of the Q/R site is detected in malignant gliomas (Maas et al. 2001). RHOQ (RAS homologue family member Q) is another mRNA subjected to A-to-I RNA editing in colorectal cancer (CRC) identified by transcriptome sequencing. The editing event changes asparagine to serine resulting in hyperactivity of RHOQ leading to cytoskeletal reorganization and invasion in CRC cell lines (Han et al. 2014).

Initially, substitution-based editing was found in mRNAs but soon it was evident that majority of the editing events occur in the non-coding region of the genome, like 5′- and 3′-untranslated region (UTR), long interspersed elements (LINEs), tRNA, Alu-RNA, miRNAs, etc. (Athanasiadis et al. 2004; Nishikura 2010). Major fraction of the editing events in ncRNA is within the Alu sequences (Carmi et al. 2011; Luo et al. 2017; Maas et al. 2006) and elevated Alu editing has been reported in multiple cancers leading to increased transcriptomic diversity (Paz-Yaacov et al. 2015). Apart from Alu there are vast majority of ncRNAs which are subject to RNA editing, one of them being miRNAs where the primary (pri) and precursor (pre) miRNA sequences as a part of their biogenesis can undergo editing.

Editing in miRNAs can influence its processing (Kawahara et al. 2007a; Yang et al. 2006) or lead to change in target repertoire (Kawahara et al. 2007b) resulting in a critical role in both healthy and diseased condition. Recently, NGS and improved bioinformatics tools have enabled the identification of multiple miRNA editing events in cancer (Alon et al. 2012). A recent study showed that ADAR2 can edit precursor of onco-miRs, like miR-222/221 and miR-21, thereby decreasing their expression into mature forms. In glioblastoma, reduced ADAR2 expression leads to increased expression of these onco-miRs, inducing proliferation and migration (Tomaselli et al. 2015). Another study identified 22 A-to-I hypoedited events in GBM patients by small RNA sequencing. Additionally, sixteen out of these 22 events were within the seed sequence and the maximum overlap of predicted targets before and after editing was only 7.53% (Paul et al. 2017), thus leading to new miRNA editing candidates for prospective therapeutic purposes. One of the identified hypoediting events (miR-376a) by this study has already been implicated in GBM both in vitro and in vivo (Choudhury et al. 2012). The unedited miRNA targets RAP2A (member of RAS oncogene family) and the edited version targets AMFR (autocrine motility factor receptor) promoting glioma cell migration and invasion (Choudhury et al. 2012). Another hypoediting event in miR-589-3p leads to redirection of target from a tumour suppressor, PCDH9 (protocadherin 9), to ADAM12 (ADAM metallopeptidase domain 12) leading to glioblastoma invasion (Cesarini et al. 2018). A recent study on 20 different cancer types has identified a RNA editing hotspot in miR-200b, where the inability of the edited version to target zinc finger E-box binding homeobox (ZEB1/ZEB2) and the ability to inhibit LIFR (leukaemia inhibitory factor receptor) promote cell invasion and migration (Wang et al. 2017).

Similar to genomic and epigenomic layers of information, transcriptome also offers a dynamic repertoire of changes followed by its physiological consequences relevant for cancer. In Fig. 18.3d, we have shown different aspects of transcriptional variations. In Fig. 18.4, a brief overview of genomics-driven identification of transcriptional mis-regulations in cancer is represented.

In the preceding sections, various features of molecular mechanisms and ways to decipher them have been discussed. We have attempted to remain focused on various signalling pathways involved in cancer. In Fig. 18.5, an overview of different signalling pathways has been presented, where the candidate genes have been elucidated using various genomic applications. The genes highlighted in the figure have been discussed in different parts of the book chapter. Obviously, to understand a holistic phenotypic dysregulation as cancer, it is important not to look at each type of molecular information in isolation. We need to integrate our data analysis approaches from various omics approaches to get a better insight into genotype-phenotype correlations. Efforts such as integrated personal omics profiles (iPOP) have shown immense potential in common and chronic diseases like type 2 diabetes (Chen et al. 2012). Such approaches are likely to become common for all chronic diseases including cancer – as health management increasingly becomes data driven.

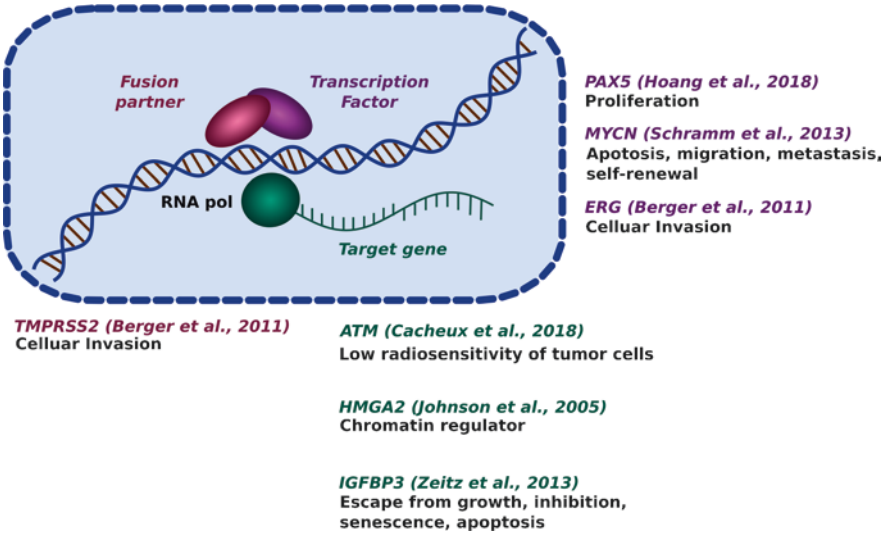

Fig. 18.4 Genomic applications in deciphering transcriptional mis-regulation in cancer. Transcription factors (purple) are often amplified, deleted, rearranged or mutated in cancer cell resulting in gain or loss of function. Chromosome translocations can generate novel fusion proteins (dark pink) with aberrant transcriptional activity. Transcription factors or fusion proteins can alter expression of target genes (dark green) and thereby contribute to the tumourigenicity. The figure highlights few genes identified by different omics techniques that cause dysregulation of transcription in cancer. For further details of the upstream mechanism refer to KEGG pathway hsa05202. (https://www.genome.jp/kegg-bin/show_pathway?map=hsa05202&show_description=show)

18.6 Future Possibilities

18.6.1 Future of Cancer Diagnostics

Genomic technologies are becoming the first line of management in cancer. As recent as in September 2018, the US Food and Drug Administration (FDA) has authorized the first next-generation sequencing-based test for acute lymphoblastic leukaemia or multiple myeloma for low-level detection of remaining cancer cells (https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm622004.htm). Many such tests are in the discovery and validation pipeline and will soon become the standard method for disease management.

As set out in the beginning of this chapter, cancer is a shift in dynamic equilibrium of cell growth and death. For a multicellular organism, under equilibrium, an aggregate of cells communicate with each other for optimum function of the whole organism. Thus for each individual cell the critical and recurrent question is whether to proliferate or not (Hesketh 2013). All normal cells rely on the rest of the organism

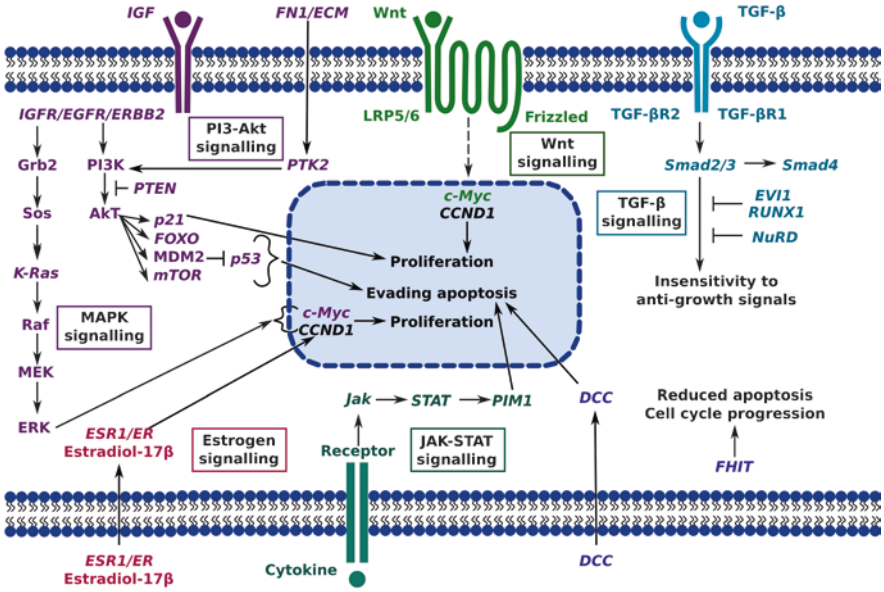

Fig. 18.5 Genomic overview of signalling pathways in cancer. Signalling pathways regulating proliferation, apoptosis, metastasis, cell cycle progression, etc., are often altered in cancerous cells. The figure highlights genes in MAPK, PI3K-AKT, Wnt, JAK-STAT and estrogen signalling pathways where alterations are identified by different NGS techniques and discussed in details in relevant sections of the chapter. For further details of the upstream mechanism refer to KEGG pathway hsa05200. (https://www.genome.jp/keggbin/show_pathway?map=hsa05200&show_description=show)

to make that decision. In cancer, individual cells become independent of such cues and thus disrupt the equilibrium. Thus to understand cancer it is important to understand the signalling cues at the single-cell level. Genomic technologies have enabled us to explore molecular wiring at individual cell resolution as well as single-molecule resolution. Now, the whole genome or transcriptome can be sequenced from a single cell (Eberwine et al. 2014) in an amplification-free manner. In the near future, such technologies will revolutionize cancer diagnosis, monitoring and therapy.

New technologies like “liquid biopsy” using cell-free nucleic acids as well as circulating tumour cells (CTCs) have added another dimension of non-invasive or minimally invasive disease diagnostic methods that have reduced invasive tumour biopsy requirements. In addition, liquid biopsies coupled with single-cell genomics have also enabled disease monitoring to have better insights into a treatment regime and disease prognosis.

18.6.2 Future of Cancer Therapy

It is estimated that half of the world population will be diagnosed with cancer in their lifetime. However, cancer survival rates are also increasing. According to the data of Cancer Research UK (https://www.cancerresearchuk.org/), more than 50% of the cancer patients now survive for 10 years or longer. Thus management of cancer is now much more efficient owing to developments in all related fields including genomics. In addition, precise genetic information, from cancer cells, is also enabling mutation-specific drug developments, known as precision medicine. Technologies like CRISPR-CAS-mediated genome editing might actually make it possible one day not too far in the future to be able to detect and correct unfavourable molecular wiring of cells to eradicate cancer.

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Applications of Computational Systems Biology in Cancer Signaling Pathways

19

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Abstract

Computational systems biology approaches to decipher cancer signaling pathways have been proposed as an essential mode to gain insight into biology of cancer cells. Pathway analysis approaches are used to discern the biological processes underlying cancer development, as it reduces the complexity, and genomic disruptions are easier to interpret in terms of biological systems. A large number of bioinformatics' tools have been developed for this purpose that can be distinctly divided based on methodology used including overrepresentation analysis, functional class scoring such as gene set enrichment analysis, single sample gene set enrichment analysis, and integrative multiple dataset-based approaches.

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The methodological challenges, limitations, and advantages of each approach are discussed, with the purpose of guiding the researchers to choose the appropriate pathway analysis method based on the available type of data and analysis tools. The various applications of pathway analytical approaches in cancer research include identifying cancer subtypes, identifying disease-associated pathways, and understanding tumor biology and biomarker identification.

Keywords
Cancer signaling pathways · Pathway analysis · Over-enrichment analysis · Gene set enrichment analysis · Network topology · Integrative pathway analysis

19.1 Introduction

Cancer cells are highly heterogeneous and complex, and the aspiration for understanding cancers based on only hyperactive oncogenes (OG) and defective tumor suppressor genes (TSG) is unlikely to fulfill the objective. A fundamental feature of cancer cells is tumor clonality. The tumor cells may have single-cell origin which subsequently begin to proliferate abnormally, migrate, and interact with other cells, suggesting that cells do not function independently but rather in collaboration with each other. Various landmark studies published by The Cancer Genome Atlas (TCGA) have shown the diversity and heterogeneity of tumors, and analyzing only sets of TSG and OG could not explain the full spectrum of cancer cells (Cancer Genome Atlas Network 2012; Cancer Genome Atlas Research Network 2014; Cherniack et al. 2017; Raphael et al. 2017). This is one reason why "the one-size-fits all" patients' approach rarely translates into effective treatment companion tests. Also, the complex repercussion at the transcriptional, translational, and posttranslational levels makes personalized treatment challenging (Wong et al. 2008). Hanahan and Weinberg described the six biological capabilities acquired by the cancer cells during cancer development as sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Hanahan and Weinberg 2000, 2011). Therefore, understanding the extraordinarily complex cancer signaling pathways and their alterations leading to the acquisition of cancer-like phenotypes is a major intellectual challenge.

Molecular information of human cancers is organized in a comprehensive atlas of the signaling networks with transcriptional and translational regulations. Studies have demonstrated that applying signaling network maps may help in assessing the effect of personalized therapies to lower clinical trial risks (Li and Mansmann 2014). In order to succeed in identifying relevant predictive or prognostic biomarkers, they should be evaluated in a multivariate and quantitative fashion based on pathway-centric approaches. There is an intricate crosstalk between pathways that works in multi-pathway networks rather than in isolation (Pawson and Linding 2008; Tan et al. 2009). Also, dysregulation of one pathway can affect the functionality of other

pathways directly (via protein-protein interactions) or indirectly (via transcriptional/translational influences). External factors like the microenvironment comprising of different cells may also impact the function of the tumor cells (Cairns et al. 2006). Hence, a pathway and network-based approach when analyzing cancer data strongly suggest the application of computational modeling approaches (Gatenby and Maini 2003; Anderson and Quaranta 2008; Barillot et al. 2012).

Methodologies for computational pathway analysis vary widely depending on the research question and the experimental data. The computational models vary ranging from simple statistical models like over-enrichment analysis based on chi-square statistics and Fisher's exact test to detailed statistical models using correlative regression like gene set enrichment analysis taking gene expression values into account to network-based models. When more than one type of molecular data is available, pathway analytical approaches using integrated dataset are possible. This allows integration of molecular information at various levels in order to understand cancer biology at multiple levels. In the following sections we will discuss the basic principles, challenges, and limitations of the different methods that are appropriate for different kinds of studies. The overview of the chapter is illustrated in Fig. 19.1.

19.2 Cancer Signaling Pathway Databases

Pathway databases serve as repositories of information about the role of genes and gene products such as proteins, mRNA, and their interactions leading to the formulation of signaling processes. In recent years thousands of molecular interactions for humans and other species have been published. However, the challenge is to mine the literature and encompass the information in a systematic manner to better understand the significance of the interactions in a given setting. Various efforts have been made to manage, integrate, and interpret the biological pathways in a meaningful manner such as Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto 2000), BioCarta (Nishimura 2001), WikiPathways (Kutmon et al. 2016), Reactome (Joshi-Tope et al. 2005; Croft et al. 2011), Nature Pathway Interaction Database (PID) (Schaefer et al. 2009), the Cancer Cell Map Initiative (CCMI) (Krogan et al. 2015), and Pathway Commons (Cerami et al. 2011), among others. The workflow to obtain the correct representation of pathways involves manual curations and data mining approaches of known literature and existing pathway databases (Adriaens et al. 2008). The steps involve retrieving biological pathway contents from curated and biological pathway databases, comparing and configuring information from different databases, literature mining, and, finally, expert curations to remove uncertain and ambiguous interactions (Adriaens et al. 2008) (Fig. 19.2). The Pathguide web interface http://www.pathguide.org/ serves as an interface of current pathway databases including 702 biological pathway-related resources and molecular interactions of which 391 are human specific (Bader et al. 2006).

The study of pathway deregulation is the key to understanding cancer biology. In order to discern the cancer biology of cells, deciphering the molecular network underlying cancers is important. One such initiative is the Cancer Cell Map Initiative

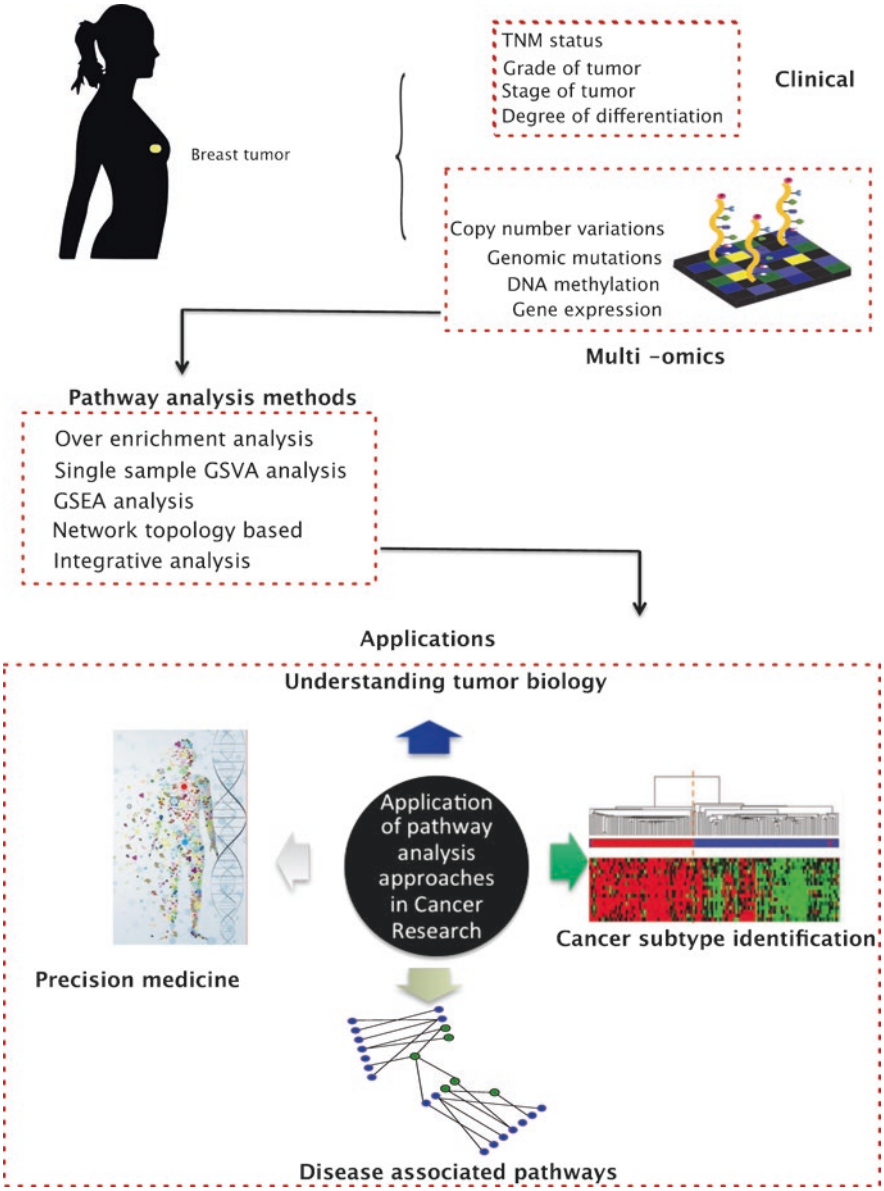

Fig. 19.1 Illustration of pathway analytical approaches and applications in cancer research

(CCMI) which focuses on elucidating molecular networks underlying cancers https://www.ccmi.org/. It generates hallmark cancer networks based on experimental and computational approaches designed to methodologically generate the complex interactions among cancer genes and the difference between disease and healthy states (Krogan et al. 2015). The interactions are manually curated and host

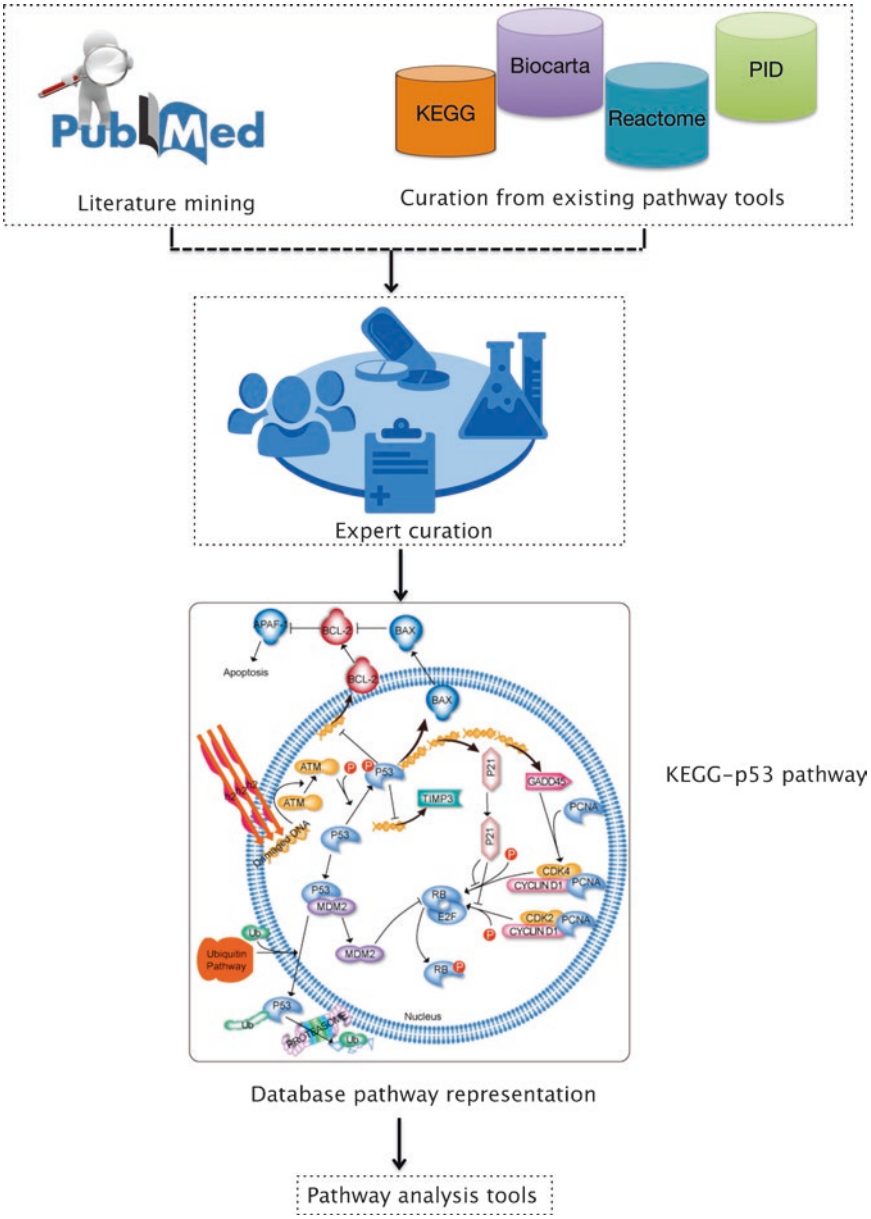

Fig. 19.2 The workflow to obtain the correct representation of pathways

ten cancer-related pathways and report approximately 100–400 molecular interactions specific to these pathways that allow mechanistic details for better understanding the different states in cancer biology. CCMI provides comprehensive information about each pathway including the types of physical interactions and cellular

locations of proteins. All the interactions can be visualized using Cytoscape infrastructure (Shannon et al. 2003) and analyzed. It also provides an open source framework NDEx http://www.ndexbio.org/ to store, share, access, and publish biological network knowledge (Pratt et al. 2017).

The most comprehensive and widely used database of gene sets for gene set enrichment analysis is the Molecular Signatures Database (MSigDB) (Subramanian et al. 2005). It incorporates 17,786 gene sets which are mainly divided into 8 collections (C1–C7 and Hallmark gene sets). It includes gene sets derived based on following derivations including gene sets representing canonical pathways from pathway sources, located on the same chromosome, sharing *cis*-regulatory motifs in their promoter, curated by computational analysis of large gene expression compendia of co-expressed modules, gene ontology (GO) terms, and oncogenic and immunologic gene sets (http://software.broadinstitute.org/gsea/msigdb). The hallmark gene sets in MSigDB are collections of more refined and concise gene sets curated by reducing redundancy and variation, thus reflecting a specific biological state or process (Liberzon et al. 2011, 2015).

The main canonical pathway databases included in MSigDB are Reactome, Kyoto Encyclopedia of Genes and Genomes (KEGG), and BioCarta. The KEGG pathway database has a collection of manually drawn pathway maps http://www.genome.jp/kegg/. It consists of 19 highly interconnected databases (Kanehisa and Goto 2000; Kanehisa et al. 2008), with genomic, chemical, and phenotypic information. The pathways are categorized into metabolic processes and genetic and environmental information processing, including signaling pathways, cellular processes, information on human diseases, and drug development (Kanehisa et al. 2008; Bauer-Mehren et al. 2009). A detailed overview of KEGG as a tool for genomic analysis describing KEGG genes and pathways is available at https://www.genome.jp/kegg/ (Aoki-Kinoshita and Kanehisa 2007). The Reactome database is an open source peer-reviewed database available at https://reactome.org/. The pathways and reaction data in Reactome are extracted from published biomedical experiments (Joshi-Tope et al. 2005; Matthews et al. 2009). The latest version (v64) of Reactome includes 2216 human pathways with 11,754 reactions curated based on 28,254 literature reviews. It covers reactions for any type of biological process and organizes them in a systematic manner. BioCarta also maps the molecular pathways and provides graphical models of molecular relationships and genomic and proteomic information. The evolution of pathway databases, limitations, drawbacks, and challenges is well described in the literature (Bauer-Mehren et al. 2009; Chowdhury and Sarkar 2015).

19.3 Pathway Analytic Approaches

19.3.1 Overrepresentation Analysis

Overrepresentation analysis (ORA) is a technique for statistically evaluating if the fractions of genes in a particular pathway are present more frequently than would be expected (*overrepresented*) in a subset of the data. The lists of genes or proteins are

selected from genome or transcriptome data often based on differential expression (up- or downregulation) in the presence of the condition and the categories of the GO terms or pathway annotations for these genes or proteins. It is also referred to as the 2×2 table method (Goeman and Bühlmann 2007).

The methodology to identify the overrepresented pathways can be divided into the following steps:

- Pathway databases:
  The pathway databases described in Sect. 19.2 can be used to map the gene sets to gene list of interest by applying pathway enrichment analysis.
- Reference gene selection:
  The reference gene list is a complete list of gene pools used when selecting the list of genes for over-enrichment analysis. Essentially, the basic idea is to assign significance to enriched pathways by comparing the fraction of genes overrepresented with the number of genes in the pathway given the selection performed from the same pool is completely random.
- Building a statistical model:
  The statistical tools model the probability whether the set of genes differentially expressed in an experiment is overrepresented in particular pathways just by chance. A number of statistical methods can be used to perform ORA including hypergeometric, $\chi 2$ (chi-square), Fisher's exact test, and binomial distribution. Further, the analysis should be corrected for multiple testing to avoid artifactual discoveries (Fig. 19.3). A number of bioinformatics tools have been developed for ORA including Onto-Express, GenMAPP (Dahlquist et al. 2002), GoMiner (Zeeberg et al. 2003), FatiGO (Al-Shahrour et al. 2004), GOstat (Beissbarth and Speed 2004), GOToolBox (Martin et al. 2004), GeneMerge (Castillo-Davis and Hartl 2003), GOEAST (Zheng and Wang 2008), ClueGO (Bindea et al. 2009), DAVID (Huang et al. 2009), WebGestalt (Wang et al. 2017), agriGO (Du et al. 2010), WEGO (Ye et al. 2006), GOFFA (Sun et al. 2006), SPIA (Tarca et al. 2009), etc. Most of these tools differ from one another based on statistical method used for over-enrichment analysis, reference genes used for analysis, and method used for correction for multiple hypothesis (Khatri and Drăghici 2005; Khatri et al. 2012).

Limitations

There are some limitations when using the ORA tools. The first limitation is the selection of genes based on arbitrary thresholds like fold change (FC >2) and/or P-value <0.05 leading to information loss. The genes, which are borderline significant with FC of 1.99 or P-value of 0.051, are not considered in ORA. Secondly, the statistical tests implemented in ORA do not take into account measured changes like probe intensities associated with each gene. Thus, all the genes are treated equally irrespective of the variable gene expression levels. Thirdly, the cooperativity of genes is ignored in ORA, where all genes are given equal weight, which in turn ignores the correlation between them. However, in biology the pathways are intercalated networks of genes where functionally related genes are often co-expressed.

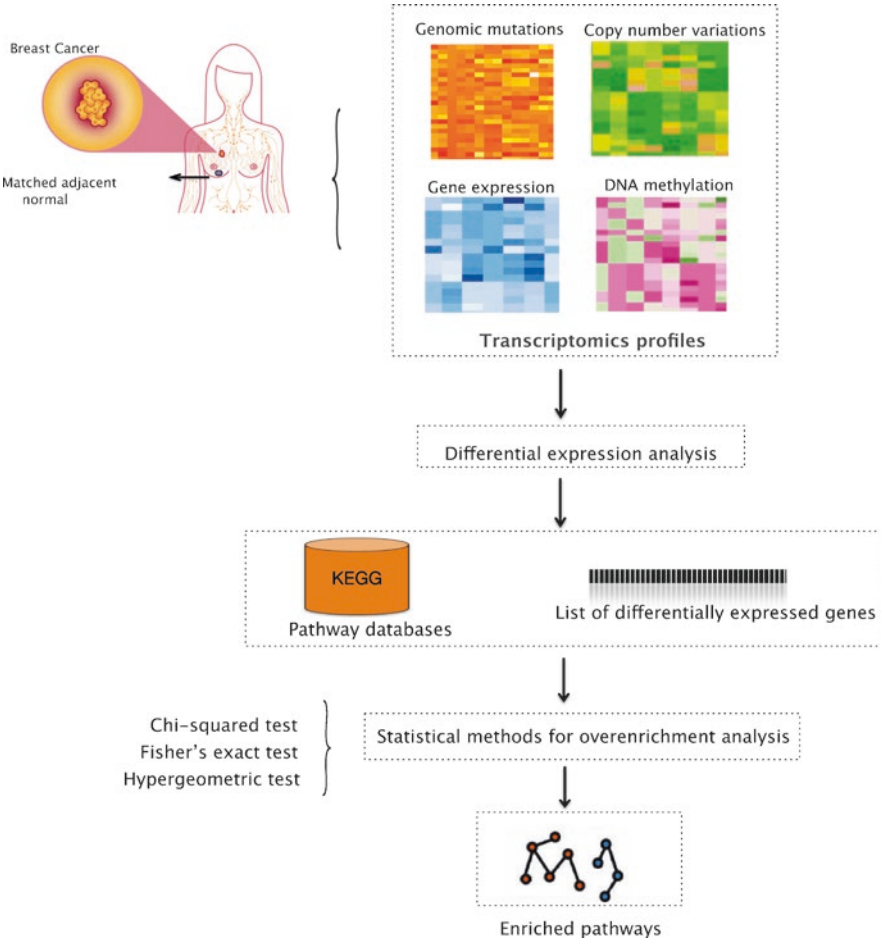

Fig. 19.3 Overview of over-enrichment analysis for pathway analysis

For instance, the two most commonly dysregulated tumor suppressors in cancer cells are the retinoblastoma protein (Rb) and p53. Both the proteins, which are co-expressed, are part of a larger network that controls the cell cycle (Ringshausen et al. 2006; Hanahan and Weinberg 2011). Fourthly, ORA does not take into account the mutual dependence between the pathways and assumes that each pathway is independent of each other. However, in biology the pathways are interlinked to each other for instance, diverse signaling pathways are responsible for epithelial mesenchymal transition (EMT) in cells, including Hedgehog, TGF-β, Wnt, and Notch signaling (Gonzalez and Medici 2014).

Case Study
Purpose: The purpose of this exercise is to identify deregulated pathways in 131 breast cancer samples vs. 131 adjacent normal samples from the METABRIC cohort (Curtis et al. 2012).

The analysis includes the following steps:

1. Excluding nonspecific genes: This step includes exclusion of genes that are not specific for the study. The nonspecific genes were selected based on the following criteria:
   - Probes with missing mapping to Entrez Gene IDs.
   - Genes with no or low variance across the samples to be informative. The variance can be used as a cutoff to select the most informative genes.
   - Duplicated probe IDs mapping to the same genes. The average of the expression of multiple probes can be used to map to the same gene ID.

In all, the filtering left 24,909 genes.

2. Defining the reference genes: This is the set of genes that defines the universe of possible genes. All the 24,909 genes were used as reference genes after nonspecific gene filtering.
3. Selecting the pathway database: The KEGG gene sets were used for pathway analysis. It incorporates 186 unique signaling pathways.
4. Identifying differentially expressed genes between breast cancer and adjacent normal samples: In order to identify differentially expressed genes between breast tumor and normal samples, we performed two-sided t-test and corrected it for multiple testing corrections using Benjamini and Hochberg correction (Benjamini and Hochberg 1995). In all, 9932 genes were statistically differentially expressed at adjusted P-value of 0.05. We used the top 1500 genes differentially expressed between the two groups based on t-statistics and adjusted P-value <0.05.
5. Identifying top deregulated pathways between breast cancer samples and adjacent normal samples: Utilizing the hypergeometric test implemented in the piano package (Väremo et al. 2013) and KEGG gene sets, 13 of the 186 gene sets were deregulated as shown in Table 19.1.

19.3.2 Gene Set Enrichment Analysis (GSEA)

The aim of gene set enrichment analysis (GSEA) is to identify pathways or sets of genes associated with a given biological phenotype. GSEA also helps elucidate underlying biological processes. It is one of the most commonly used methods for pathway enrichment analysis. This methodology uses a ranked list of genome-wide level statistics according to their association to the phenotypic groups. The main component of these gene-level statistics is that they should have a direction associated with them. The gene-level statistics can be Student *t*-stat values (obtained by

Table 19.1 The table shows the top 10 deregulated pathways in the METABRIC dataset, comparing breast cancer samples to normal samples by using over-enrichment analysis

Pathways	P-values	Adjusted P-values	Significant (in gene set)	Nonsignificant (in gene set)	Significant (not in gene set)	Nonsignificant (not in gene set)
PPAR_SIGNALING_PATHWAY	6.84E-04	2.12E-02	12	56	1488	23,353
KEGCYTOKINE_RECEPTOR INTERACTION	1.18E-03	2.74E-02	29	233	1471	23,176
WNT_SIGNALING_PATHWAY	5.83E-04	2.12E-02	20	128	1480	23,281
NOTCH_SIGNALING_ PATHWAY	6.86E-05	4.25E-03	11	35	1489	23,374
FOCAL_ADHESION	1.49E-03	3.07E-02	23	171	1477	23,238
ADHERENS_JUNCTION	5.54E-06	1.03E-03	16	57	1484	23,352
TIGHT_JUNCTION	1.92E-03	3.57E-02	17	112	1483	23,297
JAK_STAT_SIGNALING_ PATHWAY	8.93E-04	2.37E-02	20	133	1480	23,276
CIRCADIAN_RHYTHM_ MAMMAL	3.07E-03	4.39E-02	4	7	1496	23,402
ADIPOCYTOKINE_SIGNALING						
PATHWAY	5.17E-04	2.12E-02	12	54	1488	23,355

comparing samples from two conditions, say normal vs. tumor) and expression fold change value of individual genes between two groups of samples or correlation (obtained by computing between gene expression and phenotype of interest). This is carried out such that the significant genes are located at the two ends of the list. A running sum is then calculated, starting with the first gene-level statistic to the last. The sum is increased if a gene-level statistic belongs to the pathway of interest; otherwise, the sum is decreased. The enrichment score of the pathway of interest is defined as the maximum deviation from zero of the running sum. The significance is assessed in the following way: *(i)* permute the labels, say 1000 times; *(ii)* compute the enrichment score for each permutation; *(iii)* compare the enrichment score for actual data to distribution of enrichment score from permuted data. In this context, permuting the labels of the phenotypes instead of the genes maintains the complex correlation structure of the gene expression data. To control for false positives, we then perform multiple hypothesis testing. The GSEA methods analyze the gene list in context of biologically defined gene sets such as pathways or gene ontology databases and compute an enrichment score for each pathway or gene set. Thus GSEA methods provide information if a pathway or gene set is significantly enriched in one of the phenotypic groups. GSEA is available at the Broad Institute: http://software.broadinstitute.org/gsea/index.jsp. A large number of statistical methods and tools have been developed for GSEA such as *Piano (*Väremo et al. 2013*)* which can perform the GSEA (Fig. 19.4).

Limitation of GSEA

(a) Firstly, the gene-level statistics in a biological pathway does not account for gene-gene dependence and is aggregated into a single statistic.
(b) Secondly, this methodology does not consider the topology of the biological pathway.
(c) Thirdly, ranking of genes based on the gene-level statistics supersedes the strength of the statistic (although, ranking is considered to be more robust).

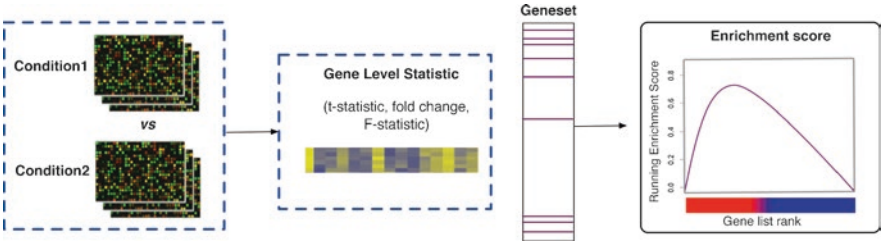

Fig. 19.4 Methodology to run GSEA. Based on the direction of differential expression, a ranked gene-level statistics is used so that significant genes are located at the two ends of the list. A running sum is then computed to obtain the enrichment score

Table 19.2 The table shows the top 10 deregulated pathways in the METABRIC dataset, comparing breast cancer samples to normal samples using GSEA

Pathway name	Stat (direction)
KEGG_OLFACTORY_TRANSDUCTION	0.37813
KEGG_PATHWAYS_IN_CANCER	0.33314
KEGG_RETINOL_METABOLISM	0.50473
KEGG_METABOLISM_OF_XENOBIOTICS_BY_CYTOCHROME_P450	0.49485
KEGG_FATTY_ACID_METABOLISM	0.62068
KEGG_DRUG_METABOLISM_CYTOCHROME_P450	0.53944
KEGG_ADIPOCYTOKINE_SIGNALING_PATHWAY	0.48990
KEGG_OXIDATIVE_PHOSPHORYLATION	−0.55609
KEGG_PYRIMIDINE_METABOLISM	−0.49637
KEGG_N_GLYCAN_BIOSYNTHESIS	−0.61024

Case Study

As a case study, we will use the METABRIC dataset and test for the enrichment of gene sets between tumor and normal samples. We used t-test to obtain gene-level statistics between tumor and normal samples. The runGSA function in the *piano* package was used to run the GSEA. Nominal P-values obtained for each pathway were corrected for multiple testing using the false discovery approach (FDR). Out of the 186 KEGG pathways, 47 pathways were found to be enriched. The top 10 pathways are presented in Table 19.2. The stat column denotes the gene set statistics for that gene set or pathway. The direction of the stat column indicates the directionality of the pathway.

19.3.3 Single Sample-Based Gene Set Enrichment Analysis

Single sample gene set enrichment analysis (SSGSEA) is another class of GSEA methods which provide information about individual samples in the group and provides scores for each sample and pathway. Counter to GSEA methods (most) SSGSEA methods are unsupervised and do not require any prior knowledge about phenotypic or biological class of the samples. Some examples of these methods are PLAGE (Tomfohr et al. 2005), Z-score approach (Lee et al. 2008), ssGSEA (Barbie et al. 2009), gene set variation analysis (GSVA) (Hänzelmann et al. 2013), and moGSA (Meng et al. 2019). As SSGSEA methods provide an enrichment score for each sample and pathway, it is possible to adjust the score for corresponding covariates of the samples. This is an advantage of SSGSEA methods compared to normal GSEA methods.

One of the early SSGSEA methods was proposed by Barbie et al. usually termed as ssGSEA (Barbie et al. 2009). In this approach, the genes are ranked using an empirical cumulative distribution function. Then an enrichment score is computed by integrating the difference between the empirical cumulative distribution function of the genes in and outside the signature. Another popular approach to perform SSGSEA is GSVA (Hänzelmann et al. 2013). The approach of GSVA is similar to

the ssGSEA method where for each pathway an enrichment score is obtained by comparing the genes that are inside and outside of the pathway. However, GSVA uses kernel estimation of a cumulative density function to rank the genes and employs Kolmogorov-Smirnov-based rank statistic to obtain the enrichment score. For ranking the genes, a kernel function is selected according to data type. For microarray data a Gaussian kernel is used while for RNA-seq data a discrete Poisson kernel is applied. Next for each pathway or gene set a Kolmogorov-Smirnov-like rank statistic (KS statistic) is calculated. To transform this KS statistic into a GSVA score (enrichment score), GSVA provides two different approaches, maximum deviation method and normalized enrichment score method. Similar to the GSEA method, maximum deviation approach computes GSVA score using maximum deviation of KS statistic from zero over all genes. The normalized enrichment score approach defines enrichment value as the difference between the minimum and maximum deviations from zero equivalent to the Kuiper test statistic (Pearson 1963; Hänzelmann et al. 2013). However, computing normalized enrichment scores require genes in a pathway (or gene set) to be annotated with "up" and "down" information. The widely used molecular signature database MSigDB does not provide directional information for gene sets (Liberzon et al. 2011). Therefore when using GSVA with MSigDB as molecular signature database, the maximum deviation approach should be used for enrichment score calculation.

Recently a new method called moGSA was proposed which can perform single sample gene set enrichment analysis while simultaneously integrating multiple types of omics datasets (Meng et al. 2019). The moGSA algorithm first uses multiple factor analysis to integrate different omics datasets and generates latent variable matrices in observation and feature space. Next the gene set matrix from a molecular signature database is projected onto the feature space matrix. The resulting matrix is multiplied by a latent variable of observation to generate the gene set enrichment score for each sample and gene set.

Limitations

The aim of the SSGSEA methods is to find differentially expressed pathways. Due to the rapid change in the high-throughput sequencing technology and batch effect, traditional gene set enrichment analysis might not be suitable. As SSGSEA methods focus on one sample at a time, they are well suited for datasets with batch effect. However SSGSEA methods need to be benchmarked to evaluate their sensitivity (smaller P-values for relevant pathways) and specificity (lower number of false positives). Moreover accommodation and analysis with multi-omics datasets is also crucial for the future. The SSGSEA methods have high potential in pathway analysis and personalized cancer medicine.

Case Study

We used the METABRIC dataset as in previous case studies and test for the enrichment of gene sets between tumor and normal samples using SSGSEA method. For analysis we used GSVA package. After computing the SSGSEA score for each pathway and sample, we used limma package to find the pathways that are

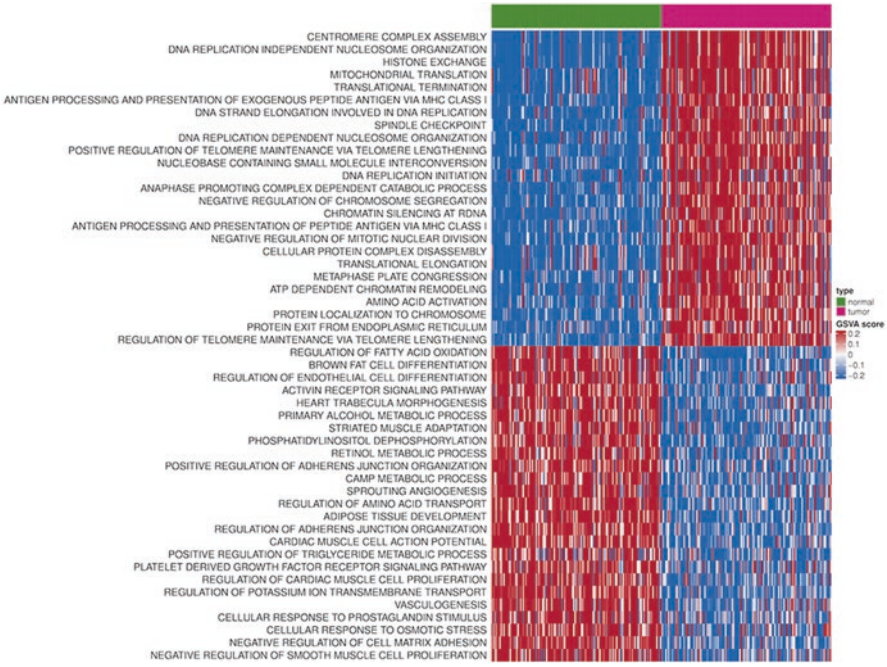

Fig. 19.5 Single sample gene set enrichment (SSGSEA) analysis for METABRIC dataset. SSGSEA was performed using R package GSVA. Heatmap shows top 25 pathways with high GSVA score in normal and tumor samples

differentially expressed in normal vs. tumor sample. Figure 19.5 shows top 25 pathways with high GSVA score in normal and tumor samples. The top bar indicates the type of sample (normal and tumor) and the heatmap represents the GSVA score for the gene set across different samples.

A virtual machine reproducing the full pathway analysis approaches discussed in this chapter is available on Code Ocean (https://codeocean.com/2018/08/17/applications-of-computational-systems-biology-in-cancer-signaling-pathways/code).

19.3.4 Integrative Pathway Analysis Approaches Across Multiple Datasets

Cancer initiation and progression is a consequence of the complex interplay of biological processes belonging to different molecular levels such as gene mutation, alteration in gene regulation networks, epigenetic changes, and changes at proteome level. Traditionally assessment of cancer-related alterations has been carried out independently on each layer of molecular information. This approach provides limited information about the biological processes and tumor characteristics. Therefore The Cancer Genome Atlas (TCGA) project is generating data at different omics

levels such as genome, transcriptome, and epigenomic data. Better translation of diverse omics data into biological knowledge and clinically useful information requires integrating multiple layers of molecular information. Several sophisticated bioinformatics methods and tools have been developed for multi-omics data analysis. These methods can be classified into three broad categories: (i) sequential combination of multi-omics data, (ii) multi-omics regression and correlation, and (iii) network-based approaches.

19.3.4.1 Sequential Combination of Multi-omics Data

In multi-omics data analysis, each data type is first analyzed separately as an independent module. In the next step multi-omics datasets are combined together to confirm and refine the findings. For example, a list of genes of interest can be produced from each dataset and then compared with each other to identify intersecting genes. Sun et al. (Sun et al. 2011) employed this approach by first finding differentially expressed and differentially methylated genes along with statistically significant copy number variation. In the next step they looked for overlaps between gene lists from different datasets (Sun et al. 2011).

Aure et al. proposed the iPAC (in-trans process associated and cis correlated) algorithm which uses a sequential analysis approach to detect cancer drivers (Aure et al. 2013). In this algorithm correlation between gene expression and copy number variation data is computed to rank the genes according to their in-cis correlation. Gene set enrichment analysis is performed on a ranked list of genes to identify phenotypic effects of the CNVs. Multi-omics data integration methods such as MCD (multiple concerted disruption) (Chari et al. 2010) and CNAmet (copy number alteration and methylation) (Louhimo and Hautaniemi 2011) are based on the assumption that expression of a gene is a function of copy number and DNA methylation. These algorithms aim to explain the gene expression variation by integrating multi-omics data. In the sequential combination of multi-omics data approaches, the order of data analysis can affect the results. For example, by first filtering genes by differential expression analysis and then analyzing their methylation status might yield a different list of genes compared to the reverse order of the analysis. Furthermore, in the sequential combination approach, a significant cutoff is needed to be defined independently for each dataset. Therefore the end results can be very sensitive to different significant cutoffs.

19.3.4.2 Multi-omics Regression and Machine Learning

This class of methods performs integrative analysis of multiple data types, to find genes or pathways which can best predict the phenotype. For example, by using linear regression, gene expression data can be combined with SNP information to find the eQTLs (Shabalin 2012; Ritchie et al. 2015; Zheng et al. 2016; Guo et al. 2016). In omics data the number of features usually supersedes the number of samples. In such cases classic linear regression is not an adequate approach. Therefore, variable selection and penalized regression techniques such as lasso, elastic net, and group lasso are preferred for multi-omics data integration. Le et al. (Le and

Bar-Joseph 2013) integrated gene expression, sequence, and protein interaction data using penalized regression to identify modules of mRNAs controlled by miRNAs. Lin et al. (Lin et al. 2013) used the group sparse correlation method to integrate SNP and gene expression data in human glioma tumors. Other examples include kernel PCA (Reverter et al. 2014; Mariette and Villa-Vialaneix 2018), regularized multiple kernel learning (Seoane et al. 2014; Speicher and Pfeifer 2015), partial least squares regression (Lê Cao et al. 2008), singular value decomposition (Berger et al. 2006; Taverner et al. 2012; Meng et al. 2014), mixed linear model (Zhang et al. 2015), Bayesian join analysis (Ray et al. 2014), and independent component analysis (Jinhua Sheng et al. 2011; Wang 2011).

19.3.4.3 Network-Based Approach

The interaction between different molecular entities in a biological system can be represented as networks. Several large databases have been developed that contain network-level information about protein-protein interaction, protein-DNA interaction, and pathways (Kanehisa 2000; Griffiths-Jones et al. 2008; Norambuena and Melo 2010; Liberzon et al. 2011; Fabregat et al. 2018). Heterogeneous omics data can be integrated by imposing it on the network of molecular interaction. Subsequently aberrant subgraphs can be identified through the network or graph-based algorithms (Ideker et al. 2002; Koller and Friedman 2009). Methods such as jActiveModules (Ideker et al. 2002) superimpose gene expression data on protein-protein and protein-DNA interaction networks. To find differentially expressed sub-networks jActiveModules uses techniques such as greedy search and simulated annealing. Another method called Graph-based iterative Group Analysis (GiGA) (Breitling et al. 2004) uses a similar principle for data integration and sub-network finding. Dittrich et al. (2008) used integer linear programming and prize-collecting Steiner tree methods to integrate the analysis of gene expression and protein-protein interaction network. Vaske et al. (2010) have developed an algorithm called Pathway Recognition Algorithm using Data Integration on Genomic Models (PARADIGM) which uses statistical graphical models with feedback loops and a Bayesian approach for data integration and biological mechanism elucidation. In PARADIGM each input gene is considered as latent variables and modeled via a belief propagation approach. It has been successfully used for the integration of gene expression, copy number variation data, miRNA, and methylation data (Vaske et al. 2010; Kristensen et al. 2012). Another method pwOmics (Wachter and Beißbarth 2015) has been designed for proteomic, genomic, and transcriptomic data integration. In this method, first the transcription factors are linked to differentially expressed transcripts, proteins, and pathways. Next, consensus graphical networks are generated using a Steiner tree or Bayesian network algorithm. Huttenhower et al. (Huttenhower and Troyanskaya 2006) have also used a Bayesian network approach for data integration and identification of network sub-modules.

Limitations

Due to the advancement in high-throughput data generation methods, multilevel omics data has become abundant. For example, TCGA provides multi-omics data

such as gene expression, copy number variation, DNA methylation, and exome sequencing from more than 10,000 tumor samples. It also has a wide range of clinical and pathological data. Given the complex nature of biological systems, integration of multi-omics data is required to understand mechanisms and to elucidate pathways. With the growing amount of data and development of new tools, it is important to compare and benchmark the methods. Methods also need to be flexible to accommodate batch or cohort differences. Integration with tools designed specifically for multi-omics data such as MultiAssayExperiment is essential for the future (Ramos et al. 2017). Furthermore, new tools are required for integrating diverse clinicopathological information such as survival or histology data with omics data.

19.4 Applications of Pathway Analytic Approaches

19.4.1 Understanding Tumor Biology

The efforts to catalogue driver genes across pan-cancer analysis have shown that only a few well-studied driver genes are frequently mutated, whereas a large number of infrequently mutated genes can also contribute to tumor biology (Tamborero et al. 2013; Buljan et al. 2018; Bailey et al. 2018). Hence to understand the mechanistic and translational changes in tumors, pathway and network analysis methods may help to improve our understanding of tumor biology. There have been tremendous efforts from the bioinformatics and systems biology perspective toward systematic assembly of molecular interactions of pathways and also toward development of pathway/network-based analytical tools.

Pathway and network analyses have been applied to cancer datasets in order to identify key regulators of cancer-related gene networks in an attempt to understand cancer mechanisms (Carro et al. 2010; Sonabend et al. 2014). It also allows to integrate various *omics* data, i.e., genomic, transcriptomic, and proteomics data, into a consolidated view of tumor biology with interpretable results in the form of perturbed biological systems such as cell proliferation and cell cycle signaling (Creixell et al. 2015). As large genomic data become available for multiple patient cohorts, higher-resolution data can be obtained for studying biological processes. However, the knowledge of tumor biology is still preliminary through the eye of pathway and network analyses as we need more accurate and extensive pathway descriptions.

19.4.2 Identification of Disease-Associated Pathways

With the advent of technological development, a large amount of transcriptomic data can be analyzed simultaneously improving our knowledge of underlying biological processes. Multiple omics data including genomics, transcriptomics, proteomics, and metabolomics can be integrated with clinical data to capture the complete picture of biological processes. Integration of these networks helps to extend our knowledge of disease signaling pathways and their interactions with

each other (Barabási et al. 2011; Califano et al. 2012). Various efforts have been made to identify disease-associated networks (Wang et al. 2010; Xiong et al. 2012); one such approach is Gene Set Association Analysis (GSAA) tool which integrates gene expression data with SNPs to identify disease-associated pathways enriched for differential expression or SNPs (Xiong et al. 2012). However, the bottleneck in identifying disease networks are the scarcity of comprehensive and detailed human interactome, lack of understanding of the biological functions, and incomplete knowledge of the role of intergenic regions of the human genome, in addition to lack of extensive epigenetic datasets (Liu and Chance 2013).

19.4.3 Cancer Classification Approaches

Cancer subtype classifiers using relevant pathways only to reduce huge variable space have allowed robust subtyping of tumors with higher accuracy (Gatza et al. 2010; Graudenzi et al. 2017). Also, the paradigm of subtyping cancers using pathway-level activity exhibits similar clinical and biological functions of subtypes which might contribute to understand the complex mechanism of cancer biology (Gatza et al. 2010), which can be further linked to therapeutic options. Top scoring pair (TSP)-based pathway analytic approach focuses on identifying smaller sets of genes in which a class is discriminated based on relative comparison of expression values of TSP of each pathway.

The basic principle of the TSP-based pathway analytical approach is a methodological extension of the "top scoring pair" (TSP) algorithm. The k-TSP algorithm exploits the gene reversal order across the classes for predicting the classes; i.e., it identifies top k-pairs where expression of gene(i) > gene(j) in one class as compared to the other class under comparison. The classifier regards these gene pairs as a good indicator of the class and uses the unweighted majority voting to predict the final class of the samples (Geman et al. 2004; Eddy et al. 2010). The two TSP-based pathway analysis approaches are gene set top scoring pairs (GSTSP) (Tan 2012) and top scoring pair pathways (TSPP) (Glaab et al. 2010). The GSTSP approach is based on two simple steps. First, given the two different biological states, it first identifies the core gene members of the pathways which are informative in distinguishing two biological states. It calculates the gene set enrichment score for each pathway, and the pathways with maximum score are defined as the most enriched gene set in the classification problem. In the second step, it constructs the classifier using the most enriched gene set that captures the core gene members in a set of IF-ELSE rules, i.e., whose relative expression levels are reversed from one state to another (Tan 2012). The authors of the GSTSP method showed the correlation of biological phenotypes of different heart failure data types and gene sets selected by GSTSP. Whereas the TSP approach is based on learning simple decision rules from pairwise comparisons of "pathway pairs" unlike "gene pairs" in the GSTSP method. It is based on five steps; first, it transforms the gene expression data into a rank matrix by sorting the expression value of each gene across the samples and

replacing the expression values with ranks. In the second step, the genes are mapped to pathway databases like KEGG, BioCarta, Reactome, etc. The genes which cannot be mapped to any of the pathways are excluded. The third step involves scoring the pathway pairs. In this step each pathway gene set rank matrix is transformed into a single vector by replacing the expression level ranks of each sample with its median value. The median rank values are then compared between pathway pairs for the biological states under comparison. Further, the TSPP pairs are identified by searching all pairs of pathways in the fourth step. In the final step the model is generated by combining TSPP decision rules into a classifier rule set which is then used for classifying other samples (Glaab et al. 2010). It has been shown that the k-TSP-based classifier approach although simple is significantly accurate and successful in classifying cancers (Tan et al. 2005; Haibe-Kains et al. 2012; Marchionni et al. 2013; Kim et al. 2016).

19.4.4 Mapping the Pharmacogenic Landscape of Precision Medicine

Cancer patients have a very different molecular mechanism and genetic makeup, which is a key to identify the correct precision medicine choices for a given patient (Hanahan and Weinberg 2000, 2011; De Palma and Hanahan 2012). These differences can be assessed by accounting the pathway activities and therapy responses. Clinically, some targeted sequencing panels have been introduced which incorporate several cancer-related genes which mainly rely on next-generation sequencing technologies for genetic screening, diagnosis, and clinical assessment (Weiss et al. 2015; Shen et al. 2015). These diagnostic panels cover genomic region of interest and may better explain the molecular alteration leading to potential deregulation of cancer pathways (Kotelnikova et al. 2016).

Systems biology approaches are important for precision oncology as each tumor has a unique genetic, epigenetic, and pathological rewiring of pathways (Barillot et al. 2012). Mathematical modeling of each tumor might help clinicians in identifying personalized treatment for patients, minimizing the toxicity and development of diagnostic biomarkers. Pharmacogenomics knowledge for personalized medicine (PharmGKB) is one such resource which provides gene-variant-drug relationship (Whirl-Carrillo et al. 2012). It maintains clinically relevant information like dosing guidelines, drug labels, gene-drug associations, and genotype-phenotype relationships (Whirl-Carrillo et al. 2012). The gene-variant-drug knowledge is further extended to the pathway information. Various algorithms have tried to implement this approach like DEAP (Haynes et al. 2013). However, there are a number of challenges when developing anticancer drugs due to the huge complexity of the cancer signaling networks. Also, the crosstalk between pathways, the reactivation of pathways by feedback inhibition mechanism, and numerous pathways controlling the cell proliferation and survival make the task of targeting anticancer drugs even more difficult (Sever and Brugge 2015).

19.5 Conclusion

This chapter describes the major pathway analytical approaches, challenges, limitations, and application of pathway analytical approaches. Pathway analysis is highly prominent in life science studies published in the literature for explaining and illustrating the biological processes underlying cancer development. Each pathway analytical approach has limitations, which are continuously addressed by leveraging advances in machine learning and computational biology. However, there remain open challenges. First discrepancies in the annotation of gene symbols, for instance, conversion from Entrez Gene IDs to Ensembl gene IDs, or incomplete annotations might result in inconsistent results depending on the choice of the pathway databases. Second, the inability to benchmark the results and to incorporate the dynamic nature of a biological system in analysis restricts the applicability of current methods. However, with improved functional annotations and technological advances, the applicability and accuracy of pathway analytical approaches will improve to better understand the complex biological systems.

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New Emerging Molecules in Cancer Research Which Hold Promise in Current Era

20

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Abstract

Advancement in technologies and insights into biological systems has steered the increase in the arsenal of anticancer drugs in the last few decades. A systematic analysis of the 'OMICS' data abetted the emergence of novel drug targets in the areas of cell signaling, tumor microenvironment modulation and immune modulation. Innovative research is aimed at therapeutic targeting of cancer by addressing molecular anomalies of tumor cells in correlation with their ability to alter tumor and immune microenvironment for a favourable clinical outcome. In this chapter we describe advances in cancer therapies with an emphasis on drugs emerging through research ideas in basic tumor biology, key signaling kinases, tumor microenvironment modulation, cancer metabolism and immunomodulation with successful therapeutic translation.

Keywords

Kinase · Immunotherapy · Tumor microenvironment · Metabolism

20.1 Introduction

Development of novel therapeutic candidates in oncology holds immense potential due to availability of exponentially growing 'OMICS' data. Conventional therapies for cancer include chemotherapy, radiation therapy and surgery that are based on an analytical and reductive approach for effective disease treatment outcome. Newer molecules in oncology are developed with a target-based approach and most of the emerging

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molecules exert a therapeutic effect by inhibition of a biological process through single molecular target engagement. Key signaling kinases have emerged as actionable drug targets. Drug screening assays with these targets have led to the approval of numerous drugs, the most exemplary being imatinib: a Bcr-Abl kinase inhibitor in chronic myelogenous leukaemia (CML). A 'synthetic lethality' screening approach that uses the concept of combination of gene targets resulting in cell lethality has revolutionized drug target screening as exemplified by the success of PARP inhibitors in BrCa-deficient cancers. Molecular medicine is now an accepted norm in cancer treatment and personalized medicine has been beneficial in several cases as evidenced by success of EGFR inhibitors erlotinib, gefitinib, etc., in non-small-cell lung cancer (NSCLC) and PARP inhibitors in ovarian cancers. However, the success of personalized medicines in oncology is overshadowed due to the complexity and continuous evolution of the disease condition in terms of emergence of newer molecular characteristics associated with the pathogenicity of the disease. In addition to targeting tumor cells, research is now aimed at exploring the pathogenicity of the disease arising out of tumor-tumor microenvironment (TME) interactions and role of immune mediators.

Biologically, the TME is a complex milieu consisting of growth promoting and inhibiting factors, nutrients (oxygen and glucose), cytokines, matrix proteins and most significantly the non-tumor cells (fibroblasts, immune cells, endothelial cells and normal epithelial cells) that surround and feed the tumor cells. Whilst transformed cancer cells harbouring gain-of-function mutations in oncogenes or depleted tumor suppressor mechanisms drive tumor growth, cancer spread is aided by association of tumor cells with non-tumor cells and other stromal components. An altered immune cell profile (infiltrating lymphocytes, cancer-associated fibroblasts (CAF), macrophages, endothelial cells) accompanied with hypoxic and nutritional stress is significant in sustaining cell proliferation and survival, evading immune surveillance, reprogramming energy metabolism and spread of cancer through metastasis. In addition, the TME also contributes to cancer recurrence and resistance to therapy. Since a highly heterogeneous tumor has the potential to adapt to or alter its' microenvironment, understanding the fundamental biology and molecular processes through which these microenvironment factors aid in cancer as well as tumor-TME interactions is critical to unravelling the mechanisms of cancer initiation, progression, non-responsiveness to therapies and identification of novel therapeutic targets.

This chapter describes advances in cancer therapies with an emphasis on drugs emerging through research ideas in basic tumor biology, key signaling kinases, TME modulation, cancer metabolism and immunomodulation with successful therapeutic translation.

20.2 Kinase Inhibitors

Phosphorylation is the most frequent form of reversible post-translational modifications that regulates intercellular communication, physiological reactions, maintenance of cellular homeostasis and modulation of immune system. Signaling through

phosphorylation is controlled via a sequential coordination of kinases and phosphatases that add and remove phosphate, respectively. Vertebrate genome is known to encode approximately 1001 protein kinases. A total of 518 functional kinases that constitute about 1.7% of all human genes have been identified so far (Manning et al. 2002). These kinases act as major signal transducers governing a milieu of cellular activities. Dysregulated protein kinases are implicated as drivers in various cancers and thus act as promising drug targets. Receptor tyrosine kinases (RTK) along with serine/threonine kinases (STK) form the bulk of targetable kinases in cancer (Arora and Scholar 2005; Takeuchi and Ito 2011). In addition to these, targeting of non-RTKs like ABL has delivered effective target inhibitors. Whilst RTKs are activated through extracellular mitogenic signals, the non-RTKs are activated by intracellular signals.

20.2.1 Receptor Tyrosine Kinase (RTK) Inhibitors

RTKs are a promising class of cell membrane receptors investigated as potential drug targets since more than three decades. RTKs mediate signal transduction from the extracellular milieu to the cytoplasm and nucleus in succession leading to activation of multiple signaling pathways. Numerous subfamilies of RTKs, such as epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGFR), hepatocyte growth factor receptor (HGFR) and anaplastic lymphoma kinase (ALK), have proven to be clinically efficacious in cancer. The deregulated activation of these kinases referred to as 'oncogene addiction' is linked to gain-of-function mutation, gene rearrangement, gene amplification, overexpression or abnormal autocrine, endocrine or paracrine stimulation of both the receptor and the ligand (Weinstein and Joe 2008). All RTKs assemble as monomers. Each monomer comprises of the N-terminus extracellular domain and the C-terminus kinase catalytic domain with a wedged-in transmembrane region. Receptor activation occurs primarily by dimerization mediated by the cognate ligands which in turn switches 'ON' the downstream signaling pathway through phosphorylation of the tyrosine motifs of specific kinase substrates. Activated RTKs lead to activation of downstream signaling cascades: PI3K/Akt/mTOR, Ras/Raf/MEK/ERK, signal transducer and activator of transcription 3 (STAT 3) and PLCγ (phospholipase C gamma)/PKC (protein kinase C) that promote proliferation, metastasis, angiogenesis, survival and spread of the tumor (Fig. 20.1) (Takeuchi and Ito 2011; Surmacz 2003). Several small molecule drugs as well as monoclonal antibodies targeting RTKs are either approved or are under clinical investigation against various cancers. The RTK inhibitors are well tolerated and clinically efficacious. However, a major problem encountered with these agents is drug resistance that the cells commonly acquire by switching to alternate signaling pathways or emergence of mutations in the kinase binding pockets. In this direction, extensive research and development efforts have led to approval of improved novel drug candidates. A few examples of RTK inhibitors that are approved or are undergoing clinical trials are given in Table 20.1 (Regad 2015).

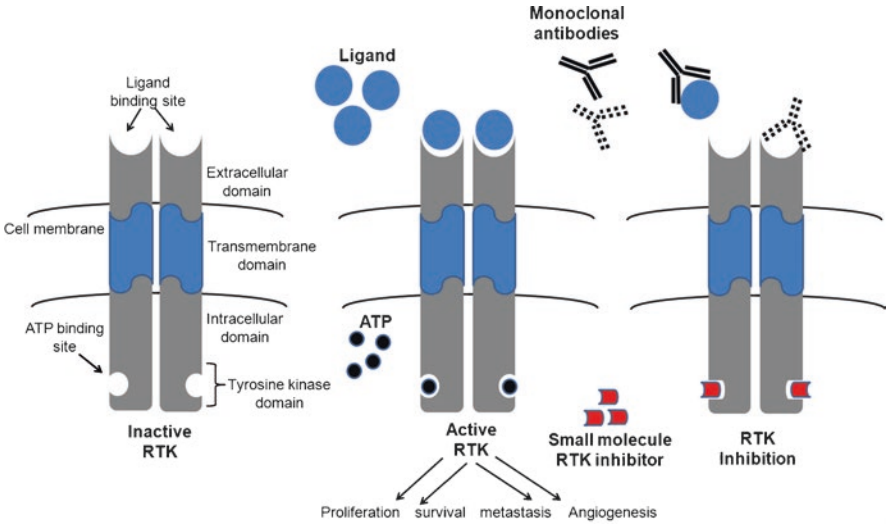


Fig. 20.1 Targeting RTK

Table 20.1 Examples of RTK targeted molecular cancer therapies (Regad 2015)

Inhibitor	Target	Approved for
Trastuzumab (Herceptin)	HER2	HER2-positive breast cancer
Cetuximab (Erbitux)	EGFR	Metastatic colorectal cancer (RAS wild type)
Panitumumab (Vectibix)		Metastatic NSCLC
Gefitinib (Iressa)	EGFR	Metastatic colorectal cancer (RAS wild type)
Erlotinib (Tarceva)		Metastatic NSCLC
Lapatinib (Tykerb)	EGFR and HER2	HER2-positive breast cancer (trastuzumab resistant)
Bevacizumab (Avastin)	VEGFR	Metastatic colorectal cancer (mCRC)

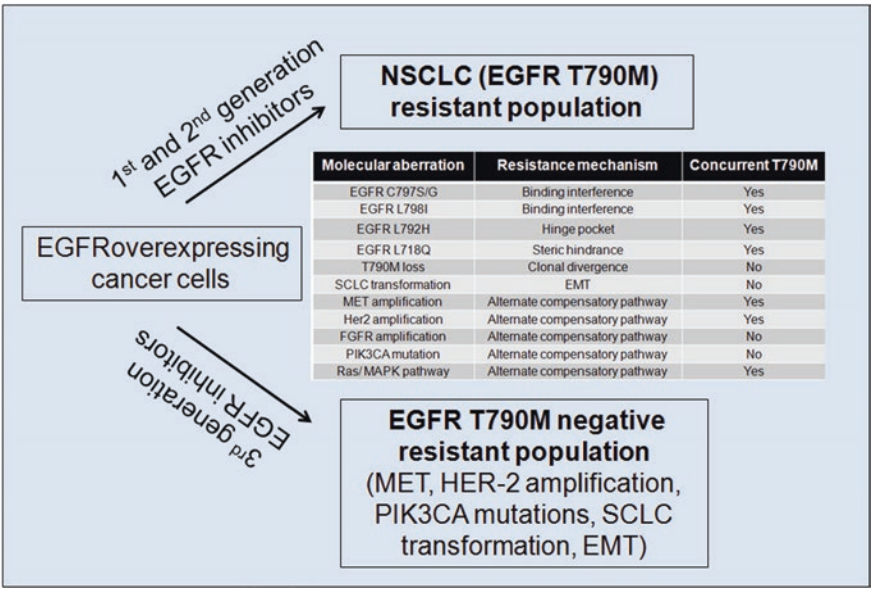
20.2.1.1 EGFR-Targeted Therapy

Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein belonging to ErbB family of tyrosine kinase receptors. EGFR oncogene-addicted cancers include head and neck carcinoma, ovarian cancer, breast cancer, NSCLC, glioblastoma, etc. (Sharma et al. 2007). The abnormal EGFR levels in cancers are linked to amplification of EGFR receptor, somatic mutations and ligand-mediated pathway modulation. Maximal clinical benefits of EGFR therapy have been observed in cancers that have (1) EGFR overexpression and (2) elevated levels of epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) that act as the natural ligands of EGFR (Putnam et al. 1992; Rusch et al. 1993). The interaction of the receptor with its' cognate ligands leads to establishment of autocrine

signaling-mediated EGFR hyperactivity. Both monoclonal antibodies and small molecule inhibitors targeting EGFR are available for clinical use. Whilst EGFR antibodies work via disruption of autocrine stimulation, suppression of catalytic kinase activity formed the basis for designing the first-generation small molecule EGFR inhibitors (Mendelsohn 1992). Monoclonal antibodies directed against EGFR ligand binding include cetuximab (Erbitux) and panitumumab (Vectibix) (Messersmith and Ahnen 2008). The first-generation catalytic EGFR kinase inhibitors include erlotinib (Tarceva) and gefitinib (Iressa). Both the drugs are reversible EGFR kinase inhibitors. These ATP-competitive inhibitors are approved in NSCLC therapy (Giaccone 2005). These drugs have been used alone or with chemotherapy (carboplatin, paclitaxel) as combination drugs (Douillard et al. 2013; Mok et al. 2009). First-generation EGFR inhibitors suffered from rapid development of drug resistance. To combat this resistance the second- and third-generation EGFR inhibitors were designed and several of these have entered the clinic in the last decade. Afatinib, dacomitinib and neratinib are classified as second-generation EGFR TKIs. They are irreversible covalent inhibitors of EGFR. In addition to EGFR they also inhibit other oncogenic ErbB kinases (HER2, ErbB3 and ErbB4) (Li et al. 2008; Engelman et al. 2007). Unlike the first-generation EGFR inhibitors, these drugs covalently bind to ErbB family members resulting in an irreversible inhibition of EGFR signaling and maintenance of persistent anti-proliferative activity. Afatinib prolongs progression-free survival (PFS) of NSCLC patients especially those carrying EGFR del19 mutations (Solca et al. 2012). In addition to NSCLC, it is being investigated in trastuzumab-nonresponsive HER2-positive metastatic breast cancer patients since it irreversibly binds to Her2, blocking its downstream signaling (Lin et al. 2012). Third-generation EGFR TKIs are designed to overcome resistance attributed to EGFRT790M mutation that results in erlotinib resistance. These kinase inhibitors are wild-type (WT) sparing. WT sparing inhibitors do not demonstrate significant inhibitory effect on WT EGFR. These include osimertinib, brigatinib, HM61713 and nazartinib (Table 20.2). These inhibitors are mutant selective as they are designed to target mutant EGFR preferentially over the wild-type EGFR. Osimertinib (AZD9291) is an oral FDA-approved EGFR TKI. It is structurally a pyrimidine-based EGFR inhibitor. It selectively inhibits the EGFR T790M or EGFR sensitizing mutation by covalently binding via the cysteine-797 residue (Cross et al. 2014).

Table 20.2 Third-generation EGFR inhibitors (Lim et al. 2018)

Inhibitor	Clinical status
Osimertinib (AZD9291)	Approved
Olmutinib (HM61713)	Development in Korea
Nazartinib (EGF816)	Phase I/II
PF0674775	Phase I/II
Brigatinib (AP26113)	Phase I/II
Avitinib (AC0010)	Phase I



Molecular aberration	Resistance mechanism	Concurrent T790M
EGFR C797S/G	Binding interference	Yes
EGFR L798I	Binding interference	Yes
EGFR L792H	Hinge pocket	Yes
EGFR L718Q	Steric hindrance	Yes
T790M loss	Clonal divergence	No
SCLC transformation	EMT	No
MET amplification	Alternate compensatory pathway	Yes
Her2 amplification	Alternate compensatory pathway	Yes
FGFR amplification	Alternate compensatory pathway	No
PIK3CA mutation	Alternate compensatory pathway	No
Ras/MAPK pathway	Alternate compensatory pathway	Yes

Fig. 20.2 Development of resistance to EGFR inhibitors. Insert table shows clinically identified mechanisms of osimertinib resistance (Lim et al. 2018)

Mechanisms of acquired resistance to EGFR inhibitors have been extensively studied. According to a clonal evolution model given by Lim et al. (Lim et al. 2018), pre-existing T790M subclone expands after first-generation EGFR inhibitor treatment whereas clones emerging after second- and third-generation EGFR inhibitor treatment acquire a variety of secondary resistant mutations that give rise to diverse molecular clones (Lim et al. 2018). Common resistance mechanisms to EGFR therapies are depicted in Fig. 20.2. EGFR T790M mutation has been reported in 50–60% cases treated with first-generation EGFR inhibitors. It is the most common secondary mutation acquired in response to first- and second-generation EGFR inhibitors. Amplifications of MET, AXL and HER2 kinases, mutations of PIK3CA, increase in small cell lung cancer (SCLC) phenotypes and epithelial-mesenchymal transition (EMT) constitute T790M-independent resistance mechanisms.

20.2.1.2 VEGFR-Targeted Therapy

The vascular endothelial growth factor (VEGF) family comprises the ligands VEGF-A, VEGF-B, VEGF-C, VEGF-D, placental growth factor (PlGF) and the receptors VEGFR-1, VEGFR-2 and VEGFR-3 (Fig. 20.3). Binding affinities of the ligands for the three receptors as well as the co-receptors that include neurolipins (NRP1 and NRP2) are variable. Most commonly overexpressed VEGF ligand is VEGF-A. VEGF121 and VEGF165 are the circulating isoforms of VEGF-A. Both these isoforms bind to the endothelial cell VEGFRs resulting in neo-angiogenesis. VEGF-A is thus a critical cytokine aiding angiogenesis. In addition to angiogenesis, VEGF signaling also promotes cell proliferation, migration, survival, vascular

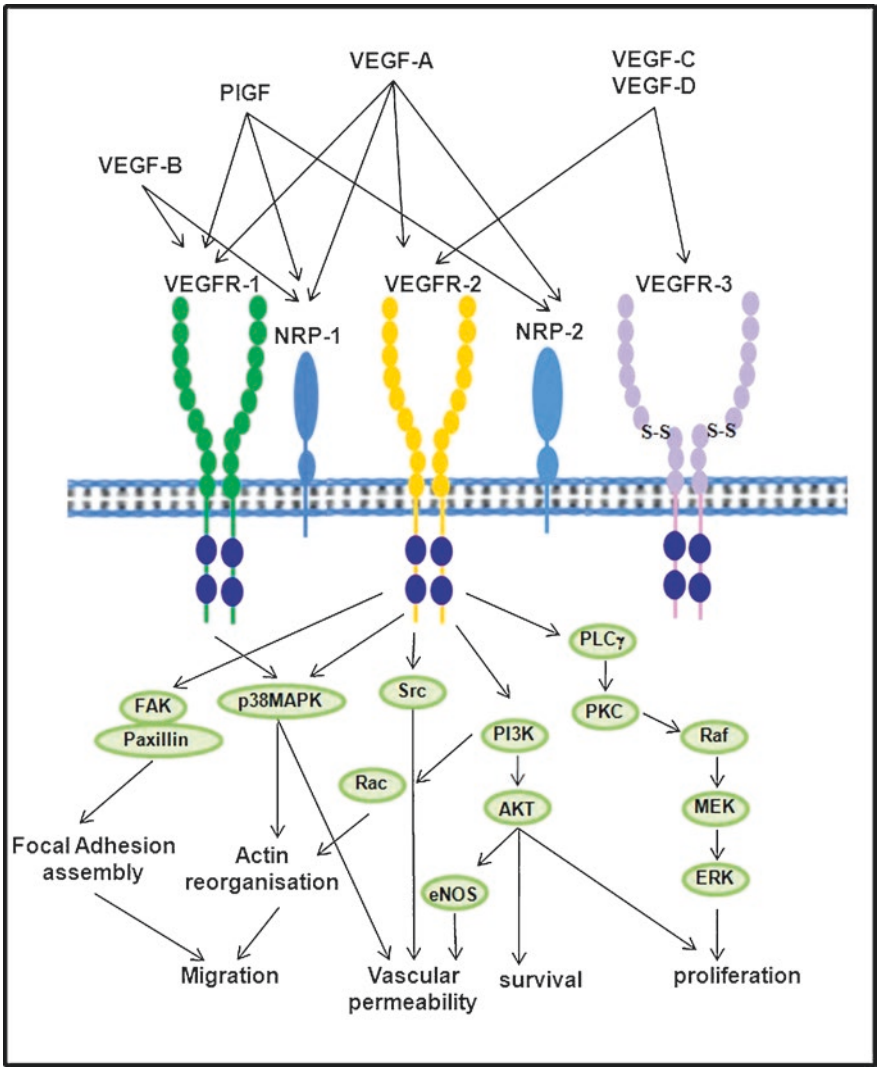


Fig. 20.3 VEGF signaling

permeability and immune modulation (Kowanetz and Ferrara 2006; Koch and Claesson-Welsh 2012). VEGF-A has been tested as a biomarker in cancer diagnosis and therapy (Karkkainen and Petrova 2002). In addition to being a key player in neo-angiogenesis, VEGF is highly immunosuppressive. It inhibits the activation of antigen-presenting dendritic cells (DCs) and effector T cell functions and promotes the immunosuppressive regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) (Yang et al. 2018a). Several studies have reported overexpression of VEGFR in several cancers and its correlation to metastasis and poor prognosis.

Anti-VEGF therapy includes monoclonal antibodies against circulating VEGF ligand (e.g., bevacizumab), receptor VEGFR-2 (e.g., ramucirumab) and numerous oral small molecule tyrosine kinase inhibitors (TKIs). TKIs with inhibitory VEGF activity include sunitinib, sorafenib, pazopanib, axitinib, regorafenib, nintedanib, cabozantinib and vatalanib. These TKIs primarily act intracellularly via suppression of catalytic kinase signaling through VEGFR-2. These are however non-specific and additionally can target other RTKs, such as PDGFR, c-Kit, CSF-R1, FLT3, etc. (Dvorak 2002; Ferrara and Adamis 2016; Kerbel 2016; Meadows and Hurwitz 2012). Newly developed antiangiogenic drugs include aflibercept, an antibody-like decoy trap agent that binds to VEGF and PDGF (Torimura et al. 2016). Monoclonal antibody bevacizumab (Avastin) was the first VEGF inhibitor approved for cancer and is currently used in colorectal, NSCLC, breast, glioblastoma and renal cancer. Except for the glioblastoma indication, all bevacizumab approvals have been in combination with chemotherapy (Ferrara et al. 2005). Ramucirumab (Cyramza) is a fully humanized immunoglobulin G1 (IgG1) monoclonal antibody that blocks VEGFR-2 and thus prevents the binding of VEGF to VEGFR. It is an approved monotherapy in advanced gastric or gastroesophageal junction adenocarcinoma patients who do not respond to first-line chemotherapy or combination therapy with paclitaxel (Fuchs et al. 2014). The list of small molecule multikinase inhibitors approved by FDA as anti-VEGFR therapy in treatment of solid tumors includes sunitinib, sorafenib and pazopanib. These inhibitors are approved as monotherapies in their respective tumor types (Zirlika and Duyster 2018). VEGF inhibitors undergoing clinical trials are listed in Table 20.3 (Zirlika and Duyster 2018; Kerbel 2008).

20.2.1.3 PDGFR-Targeted Therapy

The platelet-derived growth factor (PDGF) family consisting of ligands PDGF-A, PDGF-B, PDGF-C and PDGF-D and PDGF receptors (PDGFRs) have important functions in the regulation of cell growth and survival. PDGFR-α regulates angiogenesis both in normal and pathological conditions, mainly by stimulating the production of VEGF (Fredriksson et al. 2004). PDGFR overexpression, mutation, amplification and rearrangement are reported in numerous cancers (glioblastoma multiforme, oligodendrocytoma, oesophageal squamous cell carcinoma, GIST, etc.) (Liu et al. 2011). Several approaches have been tried for targeting PDGFR. The inhibitors include antibodies, DNA aptamers or receptor simulators that can bind to PDGF or PDGFR, thus preventing their binding to cognate ligands (Green et al. 1996; Hawthorne et al. 2008). Olaratumab, a PDGFR-α-specific monoclonal antibody, is approved for advanced soft tissue sarcoma in combination with doxorubicin (Andrick and Gandhi 2017). No specific small molecule inhibitors are available for PDGFR. However, multikinase inhibitors sorafenib, imatinib, nilotinib, etc., can suppress PDGFR activity and are effective in PDGFR overexpressing cancers.

20.2.1.4 FGFR-Targeted Therapy

Fibroblast growth factor receptor (FGFRs) family includes the receptors FGFR1, FGFR2, FGFR3 and FGFR4. Cell proliferation, migration, angiogenesis, and organ development are the major biological processes regulated through FGFR (Liang

Table 20.3 VEGF targeted therapies (Zirlika and Duyster 2018)

Inhibitor	Type	Target	Approved for
Bevacizumab (Avastin)	Monoclonal antibody	VEGF-A	mCRC, NSCLC, glioblastoma, renal cell carcinoma (RCC), epithelial ovarian cancer, fallopian tube cancer, primary peritoneal cancer, cervical cancer
Aflibercept (Zaltrap)	Recombinant fusion VEGF protein	VEGF-A, VEGF-B, PlGF	Mcrc
Ramucirumab (Cyramza)	Monoclonal antibody	Monoclonal anti-VEGFR2 antibody	mCRC, NSCLC, gastric or gastroesophageal junction adenocarcinoma
Sorafenib (Nexavar)	Multi-TKI	VEGFRs, PDGFRs, RAF, KIT, FLT3, RET	RCC, hepatocellular carcinoma (HCC), thyroid cancer
Sunitinib (Sutent)	Multi-TKI	VEGFRs, PDGFRs, FLT3, CSF1R, RET	RCC, pancreatic neuroendocrine tumors, gastro intestinal tumor (GIST)
Regorafenib (Stivarga)	Multi-TKI	VEGFRs, PDGFRs, FGFRs, TIE2, KIT, RET, RAF	GIST, mCRC, HCC
Pazopanib (Votrient)	Multi-TKI	VEGFRs, PDGFRs, FGFR1,2, c-Kit	RCC, soft tissue sarcoma
Axitinib (Inlyta)	Multi-TKI	VEGFRs, PDGFRs, c-Kit	RCC
Vandetanib (Caprelsa)	Multi-TKI	VEGFRs, EGFR, RET	Medullary thyroid cancer
Lenvatinib (Lenvima)	Multi-TKI	VEGFRs, FGFRs, PDGFRα, RET, c-Kit	Thyroid cancer, RCC
Cabozantinib (Cometriq)	Multi-TKI	VEGFRs, Tie2, cMet, AXL	Medullary thyroid cancer, RCC

et al. 2013). FGFR pathway activation can be attributed to gain-of-function mutations, receptor overexpression and chromosomal aberrations. Ligand-dependent or ligand-independent FGFR signaling in cancers can drive tumorigenesis. Both biologics and small molecule FGFR inhibitors are available. The biologics include antibodies or peptides binding to FGFR extracellular domains, thus inhibiting FGFR activation. The small molecule inhibitors are catalytic kinase domain inhibitors. The first-generation FGFR inhibitors include brivanib, E-3810, TSU-68 and BIBF1120. In addition to FGFR, these inhibitors target VEGFR and other kinases. Simultaneous inhibition of FGFR and VEGFR can inhibit both cell proliferation and angiogenesis the two important hallmarks of cancer. The second-generation inhibitors in clinical trials (BGJ398, AZD4547) are designed to selectively target FGFR (Liang et al. 2013; Brooks et al. 2011; Greulich and Pollock 2011). FGFR

Table 20.4 FGFR inhibitors in clinical trials (Liang et al. 2013)

Inhibitor	Target	Clinical status
Brivanib	VEFGR, FGFR	Phase I/II
E-3810	VEFGR, FGFR	Phase I
AZD2171	VEGFR, FGFR, KIT	Phase II
Ponatinib	FLT3, FGFR, KIT, PDGFR	Phase II
E-7080	VEGFR, FGFR, PDGFR	Phase II/III
Masitinib	KIT, PDGFR, FGFR3	Phase II
BIBF 1120	VEGFR, FGFR, PDGFR	Phase II
TSU-68	VEGFR, FGFR, PDGFR	Phase III
TKI-258	VEGFR, PDGFR, FGFR, FLT3, KIT	Phase II
BGJ398	FGFR	Phase I
AZD4547	FGFR	Phase I/II

Table 20.5 c-MET inhibitors in clinical trials (Comoglio et al. 2008; Bouattour et al. 2018)

Inhibitor	Target	Clinical status
Rilotumumab	HGF	Phase II/III
Ficlatuzumab	HGF	Phase II
Onartuzumab	c-MET	Phase II
Tivantinib	c-MET, tubulin	Phase II
Golvatinib	c-MET, KDR	Phase II/III
Cabozantinib	c-MET, KDR, RET, KIT, TIE-2, FLT3	Phase II/III
Foretinib	c-MET, KDR, RON, TIE-2, FLT3, FLT4	Phase II
Crizotinib	c-MET, ALK	Phase II
Savolitinib	c-MET	Phase II
Capmatinib	c-MET	Phase II
Tepotinib	c-MET	Phase II

inhibitors due to their non-specificity are associated with off-target toxicity. FGFR addiction across patients and tumors is highly variable. Hence prior to administering FGFR inhibitors, it becomes obligatory to evaluate FGFR levels as well as FGFR mutations for patient stratification. A list of small molecule FGFR inhibitors undergoing clinical trials is given in Table 20.4 (Liang et al. 2013).

20.2.1.5 MET-Targeted Therapy

c-MET is the receptor for the hepatocyte growth factor (HGF) that regulates proliferation, EMT, metastasis and angiogenesis. Aberrant c-Met signaling is detected in numerous cancers (colon, bladder, breast, ovarian, pancreatic, etc.). MET signaling is channelized through both autocrine and paracrine mechanisms through cognate ligands. Overexpression of Met ligands, Met receptor amplifications and gain-of-function mutation results in constitutive expression of c-Met signaling (Peruzzi and Bottaro 2006). Since the identification of the first c-MET inhibitor K252a which is a microbial alkaloid (Schiering et al. 2003), several inhibitors of MET have been tested clinically (Table 20.5) (Comoglio et al. 2008; Bouattour et al. 2018). These include both biologics and small molecule inhibitors. Monoclonal antibody

onartuzumab targets c-Met whereas ficlatuzumab and rilotumumab are designed against HGF, the c-Met ligand. Small molecule c-Met inhibitors include cabozantinib, tivantinib, foretinib, etc. Whilst savolitinib, capmatinib and tepotinib preferentially target c-Met, foretinib and cabozantinib are multikinase inhibitors hitting several kinases. ALK-positive NSCLC patients show clinical favourable response when treated with crizotinib, a dual c-MET-ALK inhibitor (Lennerz et al. 2011). Off-target toxicities and drug resistance emerging through compensatory signaling mechanisms are the major reasons for the failure of c-Met inhibitors.

20.2.1.6 c-KIT-Targeted Therapy

c-Kit or stem cell factor (SCF) is a type III RTK. Dysregulation of c-kit-mediated processes that include vital functions (fertility, homeostasis) may lead to oncogenesis. The overexpression of c-kit is observed most commonly in GIST, SM (systemic mastocytosis), AML and melanoma (Ashman 1999; Yavuz et al. 2002; Corless et al. 2004). Till date there are no c-kit-specific inhibitors. Inhibitors designed against c-kit target share inhibitory activity with other kinases. Some of these inhibitors are listed in Table 20.6 (Babaei et al. 2016).

20.2.1.7 Anaplastic Lymphoma Kinase Inhibitors

Anaplastic lymphoma kinase (ALK) is considered as an orphan RTK. It belongs to insulin receptor (IR) subfamily of tyrosine kinases. The extracellular N-terminus ALK domain comprises of low-density lipoprotein receptor domain class A (LDLa) organized between two MAM regions. Each MAM complex is an assembly of meprin, A5 protein and protein tyrosine phosphatase Mu (MAM) domain. The transmembrane glycine-rich region is followed by the intracellular C-terminus catalytic kinase domain. Secreted growth factors pleiotrophin (PTN) and midkine (MDK) are known to bind and activate ALK signaling via

Table 20.6 c-kit inhibitors (Babaei et al. 2016)

Inhibitor	Other targets	Clinical status
Axitinib	PDGFR-β, VEGF	Approved for RCC
Dovitinib (TKI-258) dilactic acid	FLT3	Phase III
Motesanib diphosphate (AMG-706)	c-RET	Phase II
Pazopanib HCl (GW786034 HCl)	FLT1, VEGF	Approved for RCC and soft tissue carcinoma
Sunitinib malate	PDGFR-β, GFR2	Approved for RCC
Masitinib (AB1010)	PDGFR-α, PDGFR-β	Phase II
Cabozantinib (XL184)	FLT3, c-RET,, Tie-2, Axl, VEGFR, c-Met, FLT1	Approved for RCC
Tivozanib (AV-951)	FLT, PDGFR-β	Phase III
Amuvatinib (MP-470)	FGFR, FLT3	Approved for AML, GIST
Pazopanib	FLT, FGFR, VEGFR, PDGFR-β,, PDGFR-α	RCC, soft tissue sarcoma
Telatinib	FLT, PDGFR-α	Phase I

mitogen-activated protein kinase (MAPK) and insulin receptor substrate 1 (IRS1) phosphorylation, respectively (Stoica et al. 2001; Lu et al. 2005). ALK activation may be independent of PTN and MDK as exemplified by long heparin chain-mediated ALK activation (Murray et al. 2015). Aberrant ALK pathway is observed in several cancers. A constitutively active ALK activity is linked to amplification, gain-of-function mutations and chromosomal translocations. Chromosomal rearrangements of ALK result in numerous fusion genes. Such rearrangements form the basis for therapeutic intervention inhibiting ALK. The fusion protein ALK comprises of the 3′ half of ALK gene and 5′ half of another gene. The 3′ half of ALK gene encloses the catalytic kinase domain whereas the 5′ half provides the promoter for ALK gene. Fusion of the ALK catalytic domain with several 5′ genes overcomes the prerequisite for a specific ligand, thus increasing the oncogenic potential of ALK. Most common fusion genes in NSCLC include NPM1-ALK and EML4-ALK (Fujimoto et al. 1996; Wang et al. 2015; Holla et al. 2017). In NSCLC, mutations in EGFR, KRAS and ERBB2 genes are mutually exclusive with ALK fusions, indicating that these genes share common downstream signaling pathways (Takahashi et al. 2010).

ALK inhibitor crizotinib is an FDA-approved drug for NSCLC patients that harbour ALK rearrangements. It is a first-in-class ALK-TKI with superior anticancer potential in comparison to platinum-based chemotherapy in ALK-rearranged NSCLC patients (Costa et al. 2015; Karachaliou et al. 2017). In addition to NSCLC, crizotinib is therapeutically efficacious in ALK-positive inflammatory myofibroblastic tumor (IMT) and anaplastic large cell lymphoma paediatric patients (Butrynski et al. 2010; Mossé et al. 2008). Resistance to crizotinib in NSCLC is an early event. However, most crizotinib resistant tumors continue to depend on ALK signaling. In this patient population second- and third-generation ALK inhibitors, such as ceritinib, alectinib, brigatinib and lorlatinib, are found to confer therapeutic benefits. In addition, indirect targeting of ALK through heat-shock protein HSP90 inhibition has been successful in suppressing ALK activity (Sang et al. 2013). A list of therapeutically sensitive ALK alterations and small molecules active against these is listed in Table 20.7 (Holla et al. 2017).

20.2.2 Non-receptor Tyrosine Kinases

Non-RTKs are found in both the cytosol and the nucleus. These enzymes catalyse the phosphorylation of tyrosine residue of the substrate proteins in response to intracellular signals. Non-RTKs regulate cellular proliferation, differentiation, adhesion, migration, apoptosis and immune responses. Thirty-two non-receptor tyrosine kinases have been identified in human cells (Manning et al. 2002). Of these, the tyrosine kinases that find application in cancer therapy include c-Abl, Src, janus-activated kinases (JAKs) and focal adhesion kinase (FAK).

Table 20.7 Therapeutically sensitive ALK alterations and small molecules active against these alterations (Holla et al. 2017)

Inhibitor	Tumor type	Sensitive variants	FDA approval for cancer indication https://www.onclive.com
Crizotinib (Xalkori)	NSCLC	EML4-ALK	ALK-positive NSCLC
	IMT	RANBP2-ALK	
		NPM1-ALK	
		ALK/F1174L	
		ALK/R1275Q	
Ceritinib (Zykadia)	NSCLC	EML4-ALK	ALK-positive NSCLC
	Thyroid	EML4-ALK/I1171T	
		EML4-ALK/V1180L	
		EML4-ALK/L1196M	
		EML4-ALK/S1206Y	
		EML4-ALK/G1269A	
Alectinib (Alecensa)	NSCLC	EML4-ALK/1151Tins	ALK-positive NSCLC
		EML4-ALK/L1152R	
		EML4-ALK/C1156Y	
		EML4-ALK/F1174L	
		EML4-ALK/L1196M	
		EML4-ALK/S1206Y	
		EML4-ALK/G1269A	
Brigatinib (Alunbrig)	NSCLC	EML4-ALK/L1196M	ALK-positive NSCLC
		NPM1-ALK/L1196Q	
Lorlatinib	Anaplastic large cell lymphoma	NPM1-ALK/C1156F	Awaiting FDA approval (August, 2018)
		NPM1-ALK/I1171T	
		NPM1-ALK/I1171N	
		NPM1-ALK/F1174I	
		NPM1-ALK/N1178H	
		NPM1-ALK/E1201K	
		NPM1-ALK/D1203N	
	Neuroblastoma	ALK/F1174L	
		ALK/F1245C	
		ALK/R1275Q	
		ALK/1151Tins	
		ALK/C1156T	
		ALK/L1196M	
		ALK/G1202R	
		ALK/G1269A	
	NSCLC	EML4-ALK/L1196M	
		EML4-ALK/G1269A	

20.2.2.1 Bcr-Abl Kinase Inhibitors

The oncogenic non-RTK Bcr-Abl results from a chromosomal translocation between chromosome 9 and 22: t(9, 22), the Philadelphia chromosome (Ph). It is a driver oncoprotein in CML. In addition to CML, 20–30% of acute lymphoblastic leukaemia (ALL) cases are found to be positive for Bcr-Abl (Burmeister et al. 2008). Clinical success of Bcr-Abl inhibitors pioneered the targeted therapy approach in cancer. Imatinib was the first clinically efficacious Bcr-Abl TKI discovered through target-based drug screening strategy (Druker et al. 1996). In spite of enjoying a high clinical success rate, imatinib resistance is a recognized clinical challenge with about one third of CML patients on imatinib therapy becoming non-responsive. Most common imatinib resistance mechanisms are increased drug efflux, reduced drug influx, activation of compensatory oncogenic mechanisms, Bcr-Abl gene amplification and acquisition of Bcr-Abl point mutations (T315I, Y253H, E255K etc.). Bcr-Abl point mutations lead to single amino acid substitutions in the kinase domain that cause conformational changes at the imatinib binding site altering the drug-enzyme interactions and hence drug resistance. T315I is the most frequently encountered imatinib resistance point mutation in CML (Hochhaus et al. 2002; Jabbour et al. 2008). To overcome imatinib resistance, new inhibitors are designed with alterations at the binding sites of the catalytic subunit without the loss of specificity. These second-generation inhibitors, nilotinib, dasatinib and bosutinib, target almost all Bcr-Abl mutations apart from T315I (Bradeen et al. 2006). Ponatinib is clinically approved third-generation TKI. It is a dual Src/Abl inhibitor that is active in T315I-mutated CML population. Other Bcr-Abl inhibitors under development include bafetinib, rebastinib, tozasertib, danusertib, HG-7-85-01, GNF-2 and 1,3,4-thiadiazole derivatives (Rossari et al. 2018).

20.2.2.2 Janus-Activated Kinase (JAK) Inhibitors

Janus kinases (JAKs) are an important class of non-RTKs as they are involved in catalysing phosphorylation events through activation by numerous cytokines, e.g. interleukin-6, interferons and various growth hormones (O'Shea et al. 2013). Structurally, JAKs comprise of a pseudokinase domain, upstream of the C-terminal tyrosine kinase domain. This pseudokinase domain of JAK is critical at maintaining a low level of tyrosine kinase activity independently of cytokine stimulation, thus leading to a constitutive phosphorylation activity. Aberrant JAK levels have been found in patients with myeloproliferative neoplasms and leukaemias (Hubbard 2018). Point mutations and amplifications are notable mechanisms associated with JAK overexpression in these cancers. The most frequently observed gain-of-function mutation is V617F mutation encountered at the pseudokinase catalytic domain (Hubbard 2018). The JAK family consists of JAK1, JAK-2, JAK-3 and TYK2 (tyrosine kinase-2). These kinases bind to the cytoplasmic region of cytokine receptors. The JAK-STAT signaling is initiated by binding of the cytokines to their cognate receptors that are located extracellularly. This binding results in receptor dimerization that in turn activates JAKs. Activated JAKs in turn activate the STAT proteins. STATs are recruited to the phosphorylated receptors through their SH2 (Src-homology 2) binding domains. Activated STATs translocate to the nucleus where

they act as transcription factors for several pro-oncogenic proteins. The JAK-STAT signaling pathway is primarily involved in organ development, tissue differentiation, homeostasis and immunity (Levy and Darnell 2002). STATs transcriptionally regulate several pro-proliferative genes, affect gene regulation through epigenetic control, induce EMT, modulate TME, and induce stem cell renewal and cellular differentiation (Groner and von Manstein 2017). Hence JAK-STAT signaling cascade inhibition is a promising strategy in cancer. Clinically approved inhibitors of JAK include tofacitinib and ruxolitinib. Tofacitinib is a pan-JAK inhibitor. It is approved for rheumatoid arthritis. Ruxolitinib is a JAK1/2 kinase inhibitor approved for polycythemia vera and myelofibrosis (Roskoski 2016).

20.2.2.3 Focal Adhesion Kinase (FAK) Inhibitors

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase. It is found localized at cell-cell adhesion junctions in the extracellular milieu. It is involved in signal transduction pathways downstream of integrins in the extracellular matrix (ECM). FAK is known to regulate cell survival, proliferation and migration (Yoon et al. 2015). Both ATP-competitive and ATP-non-competitive FAK inhibitors are under various stages of development as given in Table 20.8 (Yoon et al. 2015). The FAK inhibitors exert anticancer effects via pro-apoptotic and anti-metastatic effects. They also exhibit antiangiogenic effects. Since FAK plays critical roles in promoting invasive properties of tumor cells as well as the TME, FAK inhibitors can effectively block tumor progression, invasion and metastasis as well as modulate the TME to restore normal tissue homeostasis.

20.2.3 The Serine Threonine Kinases

Serine threonine kinases form the components of cell survival signal transduction machinery. These kinases are involved in activation of cell cycle regulators, cytoplasmic and nuclear effectors and oncogenic transcription factors. Notable amongst the targetable serine threonine kinases are the PI3K, AKT, mTOR, RAF and MEK. Both AKT/mTOR and RAS/RAF/MEK kinases are activated by the upstream RTKs and regulate myriads of oncogenic hallmarks as well as therapy resistance. Pharmacological inhibitors targeting the key kinases of the Ras-ERK and PI3K-mTOR pathways have been developed (Abrams et al. 2010; Cantley 2002). These two pathways being simultaneously activated through RTKs share a high degree of crosstalk between component proteins that may lead to differential regulation of

Table 20.8 FAK inhibitors in clinical trials

Inhibitor	Type	Target	Clinical status
Defactinib (VS-6063)	ATP competitive	FAK	Phase 2
GSK-2256098	ATP competitive	FAK	Phase I
PF00562271	ATP competitive	FAK, Pyk2	Phase1

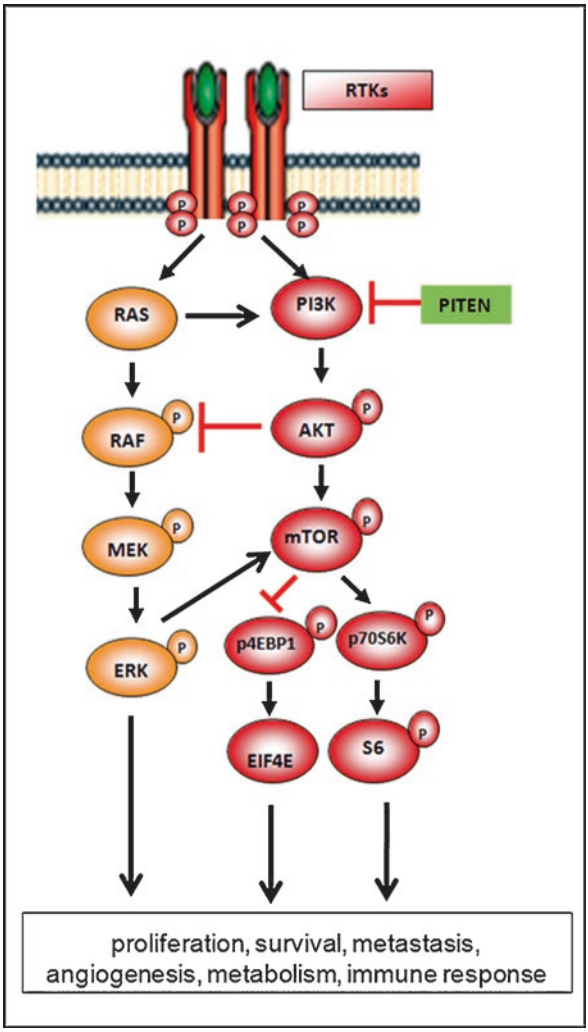


Fig. 20.4 PI3K-mTOR and Ras pathway signaling

signal transduction through the downstream cascade (Fig. 20.4). Simultaneous inhibition of these two pathways is the best strategy to overcome resistance to RTK inhibitors as well as singularly targeted RAS-RAF or PI3K pathway.

20.2.3.1 PI3K/Akt/mTOR Inhibitors

The PI3K pathway is the most dysregulated oncogenic pathway in cancer. Component proteins of this pathway (PI3K, AKT, mTOR) are druggable and small molecules targeting various components of the pathway have been developed. Genetic studies in clinical samples have shown a high prevalence of dysregulated PI3K pathway due to gain-of-function mutations and gene amplifications in its

component kinases PI3K and AKT. PTEN deletion or loss of function PTEN mutations release the brake on PI3K pathway resulting in a hyperactivated PI3K signaling.

PI3K belongs to AGC kinase family (Manning et al. 2002). The PI3K lipid kinases catalyse the conversion of phosphatidylinositol 4,5-biphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 in turn acts as a secondary messenger molecule triggering on several growth-promoting signaling cascades (Cantley 2002). There are eight mammalian PI3K enzymes that are further divided into three subclasses. The division is based on the differences of structure and substrate specificity. Of these, class I enzymes are the most commonly altered in cancer and associated with poor disease prognosis. Depending upon their regulatory subunits, the class I PI3Ks are further divided into class IA and class IB enzymes. The class IA enzymes are activated by RTKs, G protein-coupled receptors (GPCRs) and certain oncogenes such as the small G protein RAS whereas activation of Class IB enzymes is through GPCRs only (Cantley 2002; Liu et al. 2009). Both the wild-type and mutant PI3K isoform enzyme structures have been deciphered and are being utilized for drug discovery. Several molecules are reported to inhibit the different isoforms of class IA PI3K enzyme, viz. PI3K α, β, γ and δ. The most commonly expressed PI3K isoforms in solid tumors are p110α and/or p110β. The other PI3K class IA isoforms p110γ and p110δ contribute to escape of tumor cells from immune surveillance and thus promote tumor viability by modulation of leukocyte population in the tumor environment playing a significant role in TME modulation.

Since the identification of ZSTK474 in 2006, more than a dozen molecules targeting PI3K pathway have entered clinical trials (Yaguchi et al. 2006). Both pan-PI3K and PI3K isoform-specific inhibitors are now being tested in clinical trials either alone or in combination with known therapies. The isoform-specific PI3K inhibitors target disease-specific cellular processes; for example, PI3K δ inhibitor idelalisib (CAL-101) targets tumor-promoting immune mechanisms and is efficacious in B cell malignancies (Fruman and Rommel 2011). It is the first FDA-approved PI3K inhibitor and is approved for chronic lymphocytic leukaemia (CLL), small lymphocytic lymphoma (SLL) and follicular lymphoma (FL) (http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm406387). The pan-PI3K inhibitors (GDC-0941, XL147, buparlisib (BKM120), etc.) target all isoforms of PI3K and hence inhibit numerous cellular processes. A list of PI3K inhibitors that are undergoing clinical trials is given in Table 20.9 (Janku 2017). Although PI3K is a preclinically validated target, currently it remains unknown if PI3K inhibitors alone will suffice to meet acceptability in clinical trials or dual PI3K/mTOR inhibition will be required to achieve efficacy.

AKT or protein kinase B (PKB) is a serine-threonine kinase, in the AGC family, which is closely related to both protein kinase A (PKA) and protein kinase C (PKC). Other kinases that share homology with AKT kinase are the p70 ribosomal S6 kinase (p70S6k), which is downstream of mTOR in the PI3K cascade. AKT kinase is the pivotal regulatory kinase in the PI3K cascade and is highly pleotropic functionally. Functions of AKT are affected through numerous AKT target proteins which are linked to survival (BAD, MDM3, FOXO1, FOXO3A, FOXO4),

Table 20.9 PI3K inhibitors under clinical trials (Janku 2017)

Inhibitor	Target	Clinical status
Idelalisib (CAL-101)	p110γ	Approved
Buparlisib (BKM120)	Pan PI3K	Phase III
Pictilisib (GDC-0941)	Pan-PI3K	Phase II
PX-866	Pan-PI3K	Phase II
Pilaralisib (XL147)	Pan-PI3K	Phase II
Copanlisib (BAY 80–6946)	Pan-PI3K	Phase I/II
BEZ235	PI3K/mTOR	Phase I/II
GSK2126458	PI3K/mTOR	Phase I
Gedatolisib (PF-05212384)	PI3K/mTOR	Phase I
Apitolisib (GDC-0980)	PI3K/mTOR	Phase I
Apitolisib (GDC-0980)	PI3K/mTOR	Phase I
PQR309	PI3K/mTOR	Phase I/II
Alpelisib (BYL719)	p110α	Phase III
MLN1117	p110α	Phase II
Taselisib (GDC-0032)	p110β	Phase III
GSK2636771	p110β	Phase I/II
AZD8186	p110β	Phase I
SAR260301	p110β	Phase I
IPI-549	p110γ	Phase I

proliferation (CDKN1B/p27, CDKN1A/p21) and maintenance (TSC2, PRAS40) of transformed tumor phenotype. AKT also supports increased glucose uptake and angiogenesis via GSK3β and eNOS pathways, respectively (Cantley 2002; Liu et al. 2009; Engelman 2009). A major concern in developing AKT inhibitors as anticancer agents is the 'off-target toxicities'. These toxicities are primarily due to a high degree of homology in the ATP-binding pocket of AKT with PKA and PKC. A list of AKT inhibitors in clinical development is given in Table 20.10.

The protein mTOR is a direct target of AKT (Fruman and Rommel 2011; Engelman 2009). It is a serine/threonine dual kinase of PI3K-like kinase (PIKK) superfamily. mTOR is the principal metabolic regulator of protein and lipid biosynthesis. Convergence of signals for several biosynthetic and growth factor-driven cell cycle progression occurs at mTOR. mTOR constitutes two cellular complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 complex is composed of mTOR regulatory-associated protein of mTOR (Raptor), mLST8 and proline-rich Akt substrate 40 (PRAS40). It is the catalytic target of macrolide antibiotic rapamycin and hence the name. mTORC2 comprises of rapamycin-insensitive companion of mTOR (Rictor), mLST8 and stress-activated MAPK-interacting protein 1 (Sin1) (Sarbassov et al. 2005). The downstream effects of PI3K stimulation are mediated through mTOR. The first-generation mTOR inhibitors, namely, the rapalogs (everolimus and temsirolimus), are currently approved for clinical use in various cancers as listed in Table 20.11 (Wander et al. 2011). Ridaforolimus is

Table 20.10 AKT inhibitors in clinical trials

Inhibitor	Clinical status
Ipatasertib	Phase II/III (NCT03337724)
Atezolizumab (Tecentriq)	Phase III NCT02425891
Afuresertib	Phase I NCT02177682
Uprosertib (GSK2141795	Phase I NCT01902173
ARQ 751	Phase I NCT02761694
Triciribine	Phase I (NCT00363454)
MK2206	Phase II
Perifosine	Orphan drug designation for multiple myeloma and neuroblastoma

Table 20.11 Rapalogs in clinical development (Wander et al. 2011)

Inhibitor	Mechanism	Clinical status
Everolimus	Rapalog (mTORC1)	Approved
Temsirolimus	Rapalog (mTORC1)	Approved
Ridaforolimus	Rapalog (mTORC1)	Phase III
OSI-027	mTORC1/C2	Phase I
AZD8055	mTORC1/C2	Phase I/II
INK128	mTORC1/C2	Phase I

another rapalog in clinical trials. Rapalogs are allosteric inhibitors of mTOR. Preclinical studies have suggested that allosteric inhibition of mTOR by a rapalog inhibits only mTORC1 and leads to hyper-phosphorylation of AKT due to feedback loops and continued activity of mTORC2. The second-generation mTOR kinase inhibitors (TORKi) include ATP-competitive mTORC1/2 inhibitors (INK128, OSI027 and AZD8055) that are currently under clinical trials (Wander et al. 2011). Whilst resistance to mTORC1 inhibitors is mainly due to the feedback activation of AKT, the mechanism of resistance to the TORKi occurs by enhancement of intrinsic kinase activity of mTOR independent of the mutational status at the drug binding site. Thus, the second-generation of mTOR inhibitors is ineffective in tumors bearing activating mTOR mutations. Efforts are underway to develop a new class of mTOR inhibitors (third-generation mTOR inhibitors) also referred to as bivalent mTOR inhibitors that can overcome resistance to existing first- and second-generation inhibitors. In this direction, the bivalent mTOR inhibitor, RapaLink-1, possesses a unique collocation of two drug-binding pockets to allow a bivalent interaction that can overcome inhibition of the resistant mutants (Rodrik-Outmezguine et al. 2016). Due to structural homology of the kinase domains of PI3K and mTOR, several PI3K inhibitors can also effectively suppress the catalytic activity of both mTORC1 and mTORC2 kinases. These are therefore classified as dual PI3K-mTOR inhibitors and include BEZ235, GSK2126458, etc. (Table 20.9). These inhibitors are superior to mTOR inhibitors as they simultaneously inhibit both PI3K and mTOR, the two crucial signaling centres that promote cancer cell growth.

20.2.3.2 Ras/Raf/MEK/ERK Inhibitors

Ras, Raf, MEK and ERK kinases collectively referred to as the mitogen-activated protein kinase (MAPK) are serine/threonine kinases. Numerous growth factors, hormones, etc., can stimulate the activation of these kinases. Once activated these kinases transduce downstream signaling through component proteins that regulate myriads of cellular activities. Ki-Ras, N-Ras and H-Ras constitute Ras that function as GTPases to mediate GDP-GTP conversions. The sequential activation of RAS-RAF-MEK-ERK communicates signals from the extracellular milieu to the DNA. ERK is a major transcription factor involved in transactivation of numerous genes that control cell cycle progression, differentiation, metabolism, survival, migration, invasion and senescence. ERK activation is linked to several hallmarks of cancer, thus making this signaling cascade an important therapeutic target in cancer (https://am.asco.org/exploring-pathway-rasrafmekerk-pathway-fact-sheet).

With respect to pharmacological intervention, the Ras kinases are the most difficult to target. Major challenges to develop RAS inhibitors include extremely high affinity of Ras for GDP or GTP, lack of direct binding or drug docking sites on RAS, variability of tumor cells with respect to RAS dependency and feedback mechanisms of resistance (McCormick 2016). The most common post-translational modification for recruitment of RAS to the membrane are geranylation and farnesylation. However, efforts in drug designing with these approaches have not been successful. The targeted agents towards Ras kinases that inhibit farnesylation of Ras (e.g. tipifarnib, GGTI-298) and thus its binding to cell membrane have failed in clinical trials (Baines et al. 2011). RAS inhibition through therapeutic targeting of SOS protein that plays a role in GDP/GTP turnover has been tried and few small molecules that bind a unique pocket on RAS-SOS-RAS complex have been designed. This inhibition suppresses SOS-catalysed nucleotide exchange and interferes with MAPK signaling (Liu et al. 2018). $KRAS^{G12C}$, the most frequently occurring mutant KRas, was recently identified to be potentially druggable by an approach that involves allele-specific covalent binding of the drug to Cys-12 residue in the vicinity of the allosteric switch II pocket (S-IIP). This interaction inhibits the GTPase-mediated activation of Ras and hence a shutdown of its catalytic activity. Using this strategy, ARS-1620 is identified as a lead molecule representing a new generation of $KRAS^{G12C}$-specific inhibitors. It is a first-in-class, orally efficacious molecule that selectively inhibits KRAS and is well tolerated in preclinical models of drug testing. It is currently being tested in clinical trials (Matthew et al. 2018).

The other components of Ras/Raf pathway, against which inhibitors are designed and tested in clinical trials, are the Raf and MEK kinases. The first-in-class Raf kinase inhibitor developed clinically was sorafenib. Sorafenib is approved for HCC and RCC. In addition to inhibition of Raf kinase, sorafenib also inhibits angiogenesis, an important hallmark of cancer, through inhibition of PDGFR and VEGFR2 kinases (Wilhelm et al. 2008). BRAFV600E is a hotspot BRAF mutation in melanoma. Vemurafenib, an ATP-competitive RAF inhibitor approved for metastatic and unresectable BRAF-mutated melanomas, was the first BRAFV600E selective inhibitor to enter clinical trials (Bollag et al. 2012). Another BRAF inhibitor dabrafenib (Tafinlar) that inhibits mutated BRAF kinases (BRAF V600E, BRAF V600K, BRAF

V600D), as well as wild-type BRAF and CRAF kinases, received approval for BRAF V600K-mutated metastatic melanoma in 2013. RAF inhibitors vemurafenib, dabrafenib and sorafenib are catalytic ATP-competitive inhibitors. The major drawback associated with these inhibitors is the rapid development of drug resistance due to feedback activation of compensatory kinases that results from conformational changes in MAPK components such as RAF dimerization, transactivation, etc. (Poulikakos et al. 2010; Manousaridis et al. 2013; Sanchez-Laorden et al. 2014).

MEK is a tyrosine and serine/threonine dual specificity protein kinase. It recognizes and phosphorylates tyrosine and threonine residues in the Thr-X-Tyr activation loop of ERK1/2 (Downward 2003). Trametinib is the first FDA-approved MEK inhibitor and is used for BRAF V600E metastatic melanoma in 2013. Several combination strategies have been experimented clinically to derive maximal benefits from MEK inhibitors. Trametinib is being tried in combination with several therapeutic drugs, the most rational combinations being with PI3K and BRAF inhibitors (Tolcher et al. 2015). Clinically efficacious MEK inhibitor combinations include dabrafenib (Raf) with trametinib (MEK), for BRAF-V600E/K mutant metastatic melanoma and vemurafenib (Raf) with GDC-0973 (MEK) for N-RAS mutant leukaemia and BRAF mutant melanoma. Clinically, treatment with sequential drug additions of these inhibitors permits higher efficacy, less toxicity and higher inhibition of potential feedback regulatory mechanism, in comparison to chronic administration (Liu et al. 2018; Komatsu et al. 2015; Wahab et al. 2014).

ERK, the downstream effector of MEK, stimulates a wide variety of downstream substrates (MLCK, calpain, FAK and paxillin, Rho, FRA-1, etc.) (Dhillon et al. 2007). Feedback activation of ERK in KRAS-mutated cells leads to non-response to MEK inhibitors (Ahronian et al. 2015). ERK1/2 inhibitors can reverse this abnormal activation of MAPK pathway (Hatzivassiliou et al. 2012). ERK also negatively inhibits RAF. Subtle difference in the spatio-temporal activation of ERK and cross-talk between ERK with MAPK signaling counterparts and other pathways such as PI3K has been shown to critically influence the cell fate (Ramos 2008). Several ERK inhibitors are under development (Table 20.12). The most advanced ERK inhibitor in clinical development is BVD-523. It is an ATP-competitive, kinase-selective inhibitor efficacious in B-Raf inhibitor-resistant models in preclinical studies (Germann et al. 2015).

MAPK signaling kinases thus provide ample opportunity for obtaining novel anticancer molecules. Resistance to RAF and MEK inhibitors mainly arises due to

Table 20.12 ERK inhibitors in clinical trials (Liu et al. 2018)

Inhibitor	Status
LY-3214996	Phase I
LTT-462	Phase I
KO-947	Phase I
CC-90003	Phase I
GDC-0994, RG-7842	Phase I
MK-8353, SCH900353	Phase I
BVD-523, ulixertinib	Phase I/II a

gene mutations, Raf-PI3K signaling interactions and feedback control mechanisms that can be overcome with ERK inhibitors in combination with these agents in the near future.

20.3 Drugs Targeting Cancer Metabolism and Altered TME

Cancer can be considered as a metabolic disease with high input of nutrient requirement for energy needs, channelizing of the intermediates of metabolism to biosynthetic pathways that support cell proliferation and redox homeostasis. Abnormal metabolic activities of cancer cells referred to as metabolic reprogramming lead to intra-tumoral heterogeneity and tumor microenvironment changes that aid disease progression, therapy resistance as well as immunosuppression. Disturbed gradients of nutrients, oxygen and pH due to abnormal vasculature of the tumor tissue link angiogenesis, a major hallmark of oncogenesis to metabolic reprogramming. Metabolomics in cancer is thus rapidly gaining recognition on account of adaptability of cancer cells to nutrient-deficient conditions. The best characterized tumor metabolic phenotype is the Warburg effect. Warburg effect is characterized by a shift of ATP generation through oxidative phosphorylation to ATP generation through glycolysis, at normal oxygen concentrations. Reducing factor nicotinamide adenine dinucleotide phosphate (NADPH), a key cofactor produced as a result of altered cancer metabolism, participates in many enzymatic biosynthetic reactions. It functions as an antioxidant against reactive oxygen species (ROS) that alter the redox balance leading to macromolecule damage and degradation, inducing onset of cellular senescence and apoptosis. NADPH mediates removal of ROS through antioxidant molecules, viz. reduced glutathione (GSH) and thioredoxin (TRX), and thus forms part of the redox potential regulated mechanisms (Muńoz-Pinedo et al. 2012; Vander Heiden 2011). Metabolic changes are also driven by oncogenic signaling. Growth factor-mediated RTKs, RAS and PI3K pathways upregulate cellular glycolysis. Induction of SREBP and Myc oncogenes can promote lipogenesis. Glutamine addiction in tumors leads to dysregulated bioenergetic and redox homeostasis and provides precursors for many biosynthetic reactions (Choi and Park 2018). Under nutrient scarcity, AMPK activation triggers cellular metabolism to the catabolic mode. P53, a tumor suppressor, can inhibit fatty acid synthesis as well as glycolysis. Figure 20.5 depicts an interplay of the numerous metabolic factors and signaling cascades with consequent changes in the functional effects.

Metabolism is not restricted to a single cell type in the highly heterogenous tumor mass. A cooperative metabolic communication exists between tumor cells and the surrounding tissue creating changes in the TME and influencing tumor cell-stromal/immune cell interactions. For example, cancer-associated fibroblasts (CAF) upregulate the glutamine anabolic pathway to support cancer cell growth (Diaz-Cano 2012; Kleppe and Levine 2014). A voraciously exhaustive tumor metabolism impacts immune cell metabolism. In addition, a competition for nutrients and abnormal diffusion of respiratory gases leads to change in immune cell profile. For example, high levels of adenosine resulting from hypoxia are known to

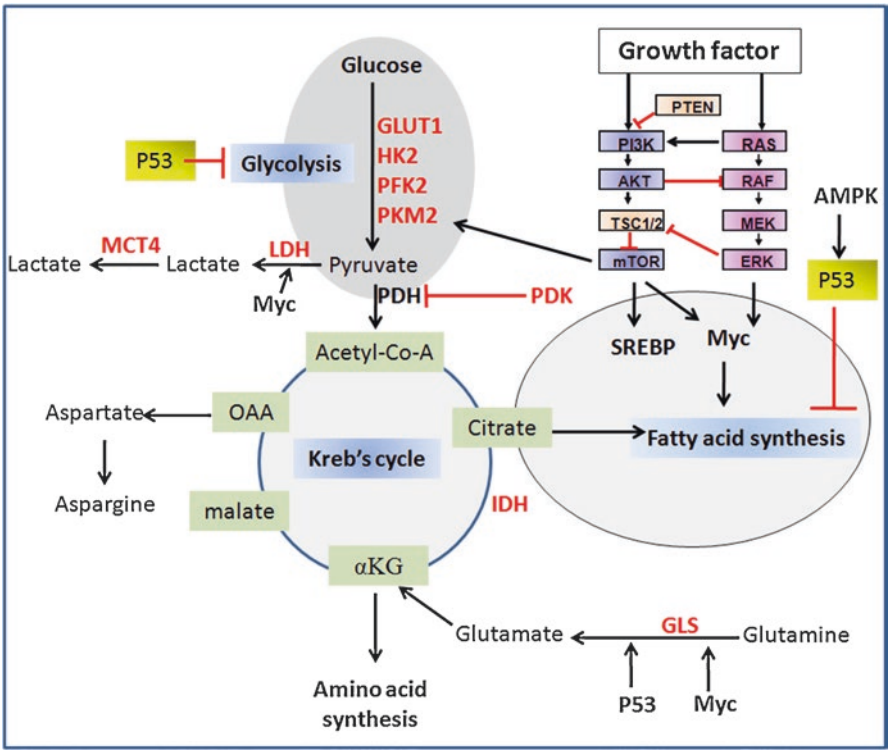


Fig. 20.5 Correlating signaling pathways with metabolic alterations in cancer cells (metabolic targets are shown in red font)

contribute to an immunosuppressed TME. Literature evidence indicates that TME supports inappropriate metabolic reprogramming. This stifles the T cell functions, balance of T cell subset populations, immune surveillance and the antitumor immune response. Metabolic alterations thus alter normal tissue physiology impacting tumor, stromal and immune cells with cells either competing or complementing each other for survival.

Tumor progression linked to metabolic reprogramming has provided several targets for cancer (Vander Heiden 2011; Kleppe and Levine 2014). Several drug targets have now emerged from metabolomics and include pyruvate kinase M2 (PKM2), phosphofructokinase (PFK), hexokinase (HK), lactate dehydrogenase (LDHA), fatty acid synthase (FAS), glutamate synthase (GLS), isocitrate dehydrogenase (IDH), TRX, etc. (Table 20.13). Molecules targeting the different metabolic nodes except IDH are still in preclinical studies. From the existing pool of clinically tested drugs, some molecules that target metabolism have been tried in patients. These include metformin, 2-dinitrophenol (DNP), 2-deoxyglucose (2DG), dichloroacetic acid (DCA) and L-asparaginase that target energy metabolism, redox potential, glucose uptake, pyruvate dehydrogenase kinase (PDK) and aspartate

pathway, respectively (Vander Heiden 2011; Choi and Park 2018; Diaz-Cano 2012; Kleppe and Levine 2014). Approved chemotherapies targeting metabolism include 5-fluorouracil (5FU) and methotrexate that target thymidylate synthase (TYMS) and dihydrofolate reductase (DHFR) enzymes, respectively. TYMS generates thymidine precursors for DNA synthesis. Similarly DHFR regulates nucleotide synthesis through folate metabolism by catalysing the conversion of dihydrofolate (DHF) to tetrahydrofolate (THF). Folate metabolism also regulates biosynthetic reactions that involve single-carbon transfers (Li et al. 1958).

The recognition of IDH mutations in AML and glioblastoma as driver mutations and subsequent approval of IDH inhibitors in cancer therapy has added to the excitement of exploring metabolomics for drug targets. IDH is a key enzyme of the citrate cycle. Wild-type IDH protein (isoforms IDH1 and IDH2) forms a homodimer that catalyses the conversion of isocitrate to α-ketoglutarate (α-KG), using the cofactor NADP. >70% of low-grade gliomas and 20% of AML carry IDH1 or IDH2 mutations. Other tumors that harbour IDH mutations include CRC, chondrosarcoma, cholangiocarcinoma and pancreatic and prostate cancer (Cairns and Mak 2013; Dang et al. 2016; Molenaar et al. 2014; Yan et al. 2009; Mardis et al. 2009; Urban

Table 20.13 Strategies to target cancer metabolism (Muńoz-Pinedo et al. 2012; Vander Heiden 2011; Diaz-Cano 2012)

	Target	Inhibitor	Status in cancer therapy
Nucleic acid synthesis	Folate metabolism (dihydrofolate reductase (DHFR)	Methotrexate, pemetrexed	Approved
	Thymidine synthesis (thymidylate synthase)	5-Fluorouracil	Approved
	Deoxynucleotide synthesis (ribonucleotide reductase)	Hydroxyurea	Approved
	Nucleotide incorporation (DNA polymerase/ribonucleotide reductase)	Gemcitabine, fludarabine	Approved
	Ribose synthesis (transketolase-like protein 1 (TKL1)	Preclinical data	Preclinical
Amino acid metabolism/ protein synthesis	Asparagine availability	L-Asparaginase	Approved
	Arginine availability	Arginine deiminase Conjugated to PEG	Phase II clinical trials
	Glutamine availability (glutaminase 1(GLS1), glutamate dehydrogenase (GDH)	CB839	Preclinical
	Phosphoglycerate dehydrogenase (PHGDH)	Preclinical data	Preclinical
Lipid synthesis	Fatty acid synthase (FASN)	Preclinical data	Preclinical
	ATP citrate lyase (ACLY)	Preclinical data	Preclinical
	Acetyl-CoA carboxylase (ACC)	Preclinical data	Preclinical

(continued)

Table 20.13 (continued)

	Target	Inhibitor	Status in cancer therapy
Glycolysis	Glucose transporters	Preclinical data	Preclinical
	Hexokinase	2-Deoxyglucose	Clinical data
	Phosphofructokinase 2 (PFK2)	Preclinical data	Preclinical
	Phosphoglycerate mutase (PGAM)	Preclinical data	Preclinical
	Pyruvate kinase M2 (PKM2)	Preclinical data	Preclinical
	Lactate dehydrogenase A (LDHA)	Preclinical data	Preclinical data only
	Monocarboxylate transporter 4 (MCT4)	Preclinical data	Preclinical
Tricarboxylic acid (TCA) cycle/ mitochondrial metabolism	Pyruvate dehydrogenase kinase (PDK)	Dichloroacetic acid (DCA)	Phase II clinical trials
	IDH1, IDH2 (isocitrate dehydrogenase)	IDHIFA (AG-221 or enasidenib)	Approved
	IDH1, IDH2	AG-120 (ivosidenib)	Phase III
	IDH1, IDH2	AG-881	Phase I
	Malic enzyme	Preclinical data	Preclinical
	Mitochondrial complex I	Metformin	Approved agent (for diabetes), clinical trials for cancer
	Pyruvate carboxylase (PC)	Preclinical data	Preclinical
Fatty acid metabolism	Monoacylglycerol lipase (MGLL)	Preclinical data	Preclinical data only
	Carnitine palmitoyltransferase 1C (CPT1C)	Preclinical data	Preclinical data only
NAD metabolism	Nicotinamide phosphoribosyltransferase (NAMPT)	FK866	Phase II clinical trials

et al. 2017). Most common IDH mutations are R132 of IDH1 and R140 or R172 of IDH2. IDH mutations confer a gain-of-function activity and are reported to be mutually exclusive. Mutated IDH converts isocitrate to D-2-hydroxyglutarate (2-HG) instead of α-KG. 2-HG is an oncometabolite that epigenetically dysregulates cellular differentiation and promotes cell proliferation. Numerous IDH inhibitors are undergoing clinical development. IDHIFA (AG-221 or enasidenib) is the first approved oral IDH inhibitor (2017) designated for the treatment of adult patients with relapsed or refractory acute myeloid leukaemia (AML) with an IDH2 mutation as detected by an FDA-approved test (https://www.businesswire.com/news/home/20170801006281/en/). AG-120 (ivosidenib) and a dual mIDH1/2 inhibitor, AG-881, are currently in phase III clinical trial for metastatic cholangiocarcinoma (NCT02989857) and phase I trial for advanced solid tumors including gliomas containing an IDH1/2 mutation (NCT02481154), respectively. Other molecules undergoing phase I clinical trials for either solid tumors or refractory AML include BAY1436032, IDH305 and Forma FT-2102 (Urban et al. 2017).

The major challenge in directly targeting metabolic pathways is the anticipated toxicity. Energy-generating metabolic pathways are common in all cells and hence it is assumed that targeting metabolic pathways in treatment of cancer would adversely affect the normal tissues. However, the success of folate metabolism inhibitors proves otherwise. Inhibitors of these targets have proven to be clinically efficacious without overt toxicity. This indicates that a therapeutic window exists for metabolism targeting anticancer drugs. For metabolic adaptability to a stressful nutrient-deficient TME, cancer cells depend on specific metabolic pathways such as glutamine addiction, increased glycolysis, etc. These pathways in turn may be linked to the oncogenic signaling pathways (Fig. 20.5). Identification of tumor-specific metabolic reprogramming would be the limiting factor in successful translation of metabolism targets.

20.4 Immunotherapies

Tumor cells exhibit a variety of features to evade the immune response (Fig. 20.6). These include lack of co-stimulatory surface molecules such as B7 for antigen presentation resulting in T cell anergy, low expression of MHC class I or class II receptors, expression of FasL (Fas ligand), FasL-mediated apoptosis of lymphocytes, secretion of inhibitory cytokines such as TGFβ, expression of mucins that mask their tumor-specific antigens, etc. The major effectors of antitumor immune response are the T cells and natural killer (NK) cells. The T cells are highly effective against tumors associated with strongly immunogenic stimulators like oncogenic viruses or ultraviolet radiations. Weakly immunogenic tumors escape T cell surveillance. Cytotoxic T cells (Tc) mediate tumor cell lysis through release of granzymes, perforins or tumor necrotic factors (TNF) (Fig. 20.6). NK cells attack MHC class I-negative tumor cells eliciting the non-specific MHC I-independent tumor cell lysis (Rabson et al. 2005). In addition to the characteristic phenotypic

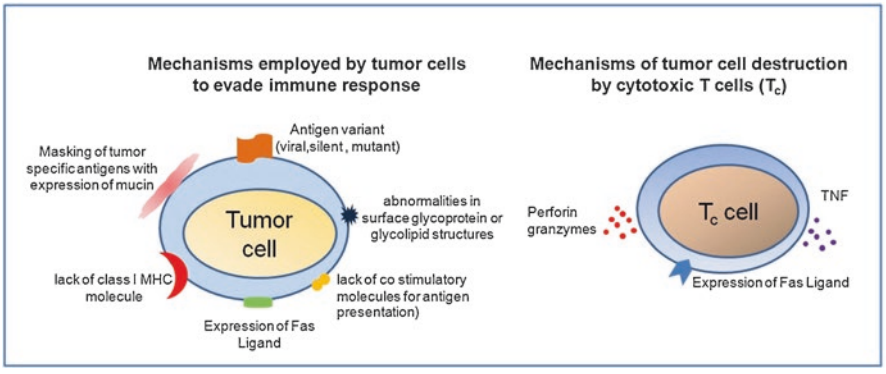


Fig. 20.6 Tumor-immune cell interactions

features described above, tumor cells evade antitumor immune attack through activation of immune checkpoints (CTLA-4, PD1, PDL-1) (Chen and Mellman 2017). The development and clinical success of anti-CTLA4, anti-PD-1 and anti-PDL1 monoclonal antibodies in solid tumors has solicited the inclusion of these immunotherapies in cancer treatment and several such inhibitors are in clinical trials (Zou et al. 2016).

TME appears to influence immunotherapy. Chronic inflammation may induce tumor immunosuppression, restrict T cell proliferation and aid in tumor survival, proliferation and angiogenesis. Cancer-related inflammation contributes to immune evasion and pro-proliferative TME, and hence new therapeutic options customized to interfere with inflammation in the TME are being tested. These strategies include TME modulation through metabolic targeting, inhibiting inflammatory cytokines, blocking the recruitment of myeloid-derived suppressor cells (MDSCs) and T regulatory cells (Treg) and modulating immunosuppressive functions.

20.4.1 Antigen-Independent Cytokine Therapy

Use of cytokine therapy in melanoma and kidney cancer pioneered immunotherapy in cancers. Cytokine-mediated immune responses are highly complicated as a homeostasis of various cytokines needs to be maintained. The reaction is highly non-specific and results in activation of a variety of T cells, NK cells and macrophages. Immunomodulatory cytokines are important as an adjuvant therapy in cancers in spite of the observed toxicity. Clinical benefits observed with this approach include administration of interleukin-2 (IL-2) in renal cell carcinoma, IFNγ and IFNβ in the hairy cell leukaemia and high-risk melanoma (Nakamura and Smyth 2017).

20.4.2 Cancer Vaccines

Cancer vaccines can be grouped into four types: (1) peptide vaccines, (2) cellular vaccines, (3) viral vector vaccines and (4) nucleic acid vaccines. Cancer vaccines aid antitumor immune surveillance via induction of new antigen-specific T cell responses as well as augmentation of innate immune responses. Because of its ability to induce and potentiate tumor-specific immune response, a cancer vaccine can be potentially combined with immune checkpoint inhibitors or other immune therapeutics without a high risk of autoimmunity.

20.4.2.1 Peptide Vaccines

Peptide vaccines targeting tumor mono-antigens have not been highly successful as observed for adjuvant trials of the peptide vaccine targeting MAGE-A3 in melanoma and NSCLC and failure of tecemotide (liposomal BLP25 (L-BLP25)) and a peptide vaccine that targets MUC-1 in phase III trial that compared tecemotide with placebo as a maintenance therapy in patients with unresectable stage III NSCLC. Another peptide vaccine in clinical development is rindopepimut that consists an

EGFRvIII peptide conjugated to keyhole limpet hemocyanin (KLH). Randomized phase II trials with this peptide in relapsed glioblastoma (ReACT) appear to show promising clinical benefits (Subramaniam et al. 2016).

20.4.2.2 Tumor Cell Vaccines

Tumor cell vaccines are made from patient cancer cells. It can be autologous or allogenic. OncoVAX®, developed by Vaccinogen, is an example of targeted, patient-specific autologous therapy for treatment of occult disease in early stage colon cancer (Hanna 2012). Belagenpumatucel-L (Lucanix) is an allogeneic tumor cell vaccine. Lucanix consists of four irradiated NSCLC cell lines that have been modified with transforming growth factor-β2 (TGF-β2) antisense gene plasmid (Giaccone et al. 2015).

20.4.2.3 Immune Cell Vaccines

These include dendritic cell vaccine, an example being sipuleucel-T (Provenge), targeting mono-antigens in prostate cancer. Sipuleucel-T is an active cellular immunotherapy that is obtained by co-culturing autologously harvested peripheral blood mononuclear cells (PBMCs) containing antigen-presenting cells (APCs) with PA2024 (prostatic acid phosphatase-granulocyte macrophage colony-stimulating factor (GM-GSF) conjugate), which serves as an immune cell activator (Madan and Gulley 2011).

20.4.2.4 Nucleic Acid-Based Vaccines

Cancer cells contain tumor-associated antigens. Nucleic acid-based vaccines are designed to specifically target these antigens in association with co-stimulatory molecules of the immune system. Both recombinant DNA and RNA vaccines are under development (Guo et al. 2013). These vaccines can potentially deliver multiple antigens and are not restricted by the patient's HLA type. Co-stimulatory molecules such as CD40L, CD70, Ox40L and GITR can be incorporated into mRNA-based vaccines to enhance their immunogenicity (Ulmer et al. 2012; Coban et al. 2011).

20.4.3 Oncolytic Virus Therapy

Immunotherapy with oncolytic viruses is a novel approach in cancer treatment. In this therapy native or engineered viruses that selectively infect and kill cancer cells are used. These mainly act via acute tumor cell infection and lysis by the virus leading to tumor debulking (Kaufman et al. 2015). Oncolytic viruses also provide immense development scope for cancer therapy as the viral genome can be altered to enhance antitumor immunity and promote tumor-targeting effector T cell activation signals. Oncolytic viruses are designed to attenuate viral growth and lytic activity, enhance favourable cytokine response and promote infiltration of cytotoxic T

cells and NK cells (Kaufman et al. 2015). Talimogene laherparepvec (T-VEC) is a first-in-class oncolytic virotherapy to receive FDA approval against advanced melanoma. It is a modified oncolytic herpes simplex virus type 1 in which two infected cell protein (ICP) genes are replaced by the coding sequence for the cytokine GM-CSF. Enhanced local expression and secretion of GM-CSF favours antigen-presenting cell (dendritic cells) recruitment to the tumor microenvironment, thereby promoting the induction of antitumor immunity. Further, ICP deletion in T-VEC induces viral replication, enhances antigen presentation and increases oncolytic therapeutic activity (Farkona et al. 2016). Other oncolytic virus cancer vaccines under development include human papillomavirus for cervical cancer and hepatitis B virus for hepatocellular carcinoma (Finn 2018).

20.4.4 Therapy with Monoclonal Antibodies

Monoclonal antibodies targeting growth factor receptors on tumor or endothelial cells (Herceptin, bevacizumab, etc.) are effective immunotherapies. Monoclonal antibodies linked to toxin or radionuclide effectively kill tumor cells. Immunotoxins include anti-idiotype antibody conjugated with ricin and anti-lymphoma drug (Zevalin or Bexaar) that employs a monoclonal anti-CD20 antibody attached to radioactive 90Yor 131I. Bispecific antibodies are another promising strategy in cancer immunotherapy. A bispecific antibody recognizes and binds two different antigens simultaneously. Currently, two bispecific antibodies, catumaxomab (anti-EpCAM and anti-CD3) and blinatumomab (anti-CD19 and anti-CD3), have been approved for patient care. A list of bifunctional antibodies undergoing clinical trials is listed in Table 20.14 (Chena et al. 2016).

20.4.5 Adoptive T Cell Therapies

Adoptive T cell therapies harness the tumor-killing properties of T cells. T cells are genetically engineered to express tumor-specific recognition receptors that include both conventional T cell receptors (TCRs) and lab synthesized constructs referred to as chimeric antigen receptors (CARs) (Hinrichs and Rosenberg 2014; Harris and Kranz 2016). TCRs and CARs differ in their immune signaling properties and sensitivity to antigens. TCRs target the MHC-bound cancer-associated antigens. CARs bind to an extracellular, antigen recognition molecule that comprises an antibody domain, a stalklike region, a transmembrane region and intracellular signaling domains derived from proximal T cell signaling machinery (Fig. 20.7) (Harris and Kranz 2016). Thus intracellular MHC-linked peptides serve as targets for TCR whilst CARs are designed to target cell surface antigens, e.g. GPC3 as in hepatocellular carcinoma cells. TCR engineering aims towards augmenting the affinity of immune T cells against the same class I peptide MHC antigen.

Table 20.14 Bifunctional antibodies undergoing clinical trials (Chena et al. 2016)

Name	Targets	Indication
Catumaxomab	EPCAM, CD3	Malignant ascites (approved)
		Ovary cancer (phase II)
		Gastric cancer (phase II) epithelial cancer (phase I)
Lymphomum (FBTA05)	CD20, CD3	BCL (phase I)
Ertumaxomab	HER2, CD3	Metastatic breast cancer (phase I)
Blinatumomab (AMG 103)	CD19, CD3	ALL (approved)
		ALL relapsed/refractory (phase II)
		DLBCL (phase II)
		NHL (phase I)
Solitomab (AMG 110)	EPCAM, CD3	Colorectal cancer (phase I)
AMG 211 (MEDI-565)	CEA, CD3	Gastrointestinal cancers (phase I)
MT 112 (BAY2010112)	PSMA, CD3	Prostate cancer (phase I)
MGD006	CD123, CD3	AML (phase I)
MGD007	gpA33, CD3	Colorectal cancer (phase I)
AFM11 T cell	CD19, CD3	Non-Hodgkin's lymphoma (phase I)
AFM13	CD30, CD16	Hodgkin's lymphoma (phase I)
IMCgp100	Gp100, TCR	Malignant melanoma (phase II)
		Melanoma (phase I)
rM28	MAPG, CD28	Metastatic melanoma (phase II)

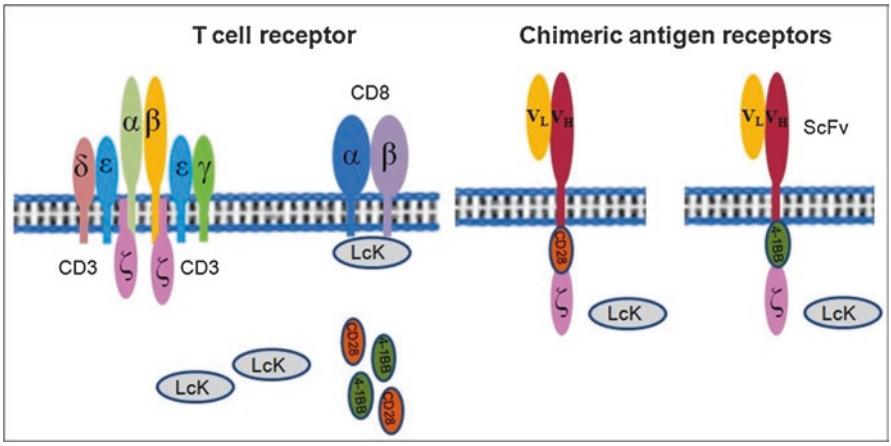


Fig. 20.7 T cell receptor (TCR) and chimeric antigen receptor (CAR) (Harris and Kranz 2016)

Non-target TCR peptide affinity is reduced to avoid cross-reactivities. CARs are MHC-independent receptors. Structural components of CAR include single-chain variable fragment (scFv) recognition domain capable of binding to cell surface antigens, intracellular signaling domains from CD3ζ and a co-stimulatory

molecule (typically CD28 or 4-1BB). Signaling is initiated by lymphocyte-specific protein tyrosine kinase (Lck)-mediated phosphorylation of immuno-tyrosine activation motifs (ITAMs) within the cytoplasmic domains of CD3 (Harris and Kranz 2016). Each CART production is a technically challenging process. Designing of each CAR requires maintenance of antigen specificity, 3D confirmation affinity and binding properties of the scFv recognition domain and intracellular signaling domain(s) that induce T cell polyfunctionality and persistence. For example, CD19-reactive CAR designed for advanced chronic lymphocytic leukaemia (CLL) is composed of a fusion protein between extracellular single-chain anti-CD19 antibody, the transmembrane domain, 4-1BB (CD137), and the CD3ς chain signaling motif. Autologous T cells transduced with the CD19-reactive CAR have shown potent clinical activity against 100% (3/3) CD19+ patients with advanced CLL (Shi et al. 2014). A list of solid tumor antigens being targeted using CART cell therapy is given in Table 20.15 (Mirzaei et al. 2017). Use of adoptive cell transfer is highly complex but recent progress has been promising and may revolutionize the field of cancer immunotherapy.

20.4.6 Immune Checkpoint Inhibitors

Antitumor immune response is a tightly regulated process. The response is initiated by antigen processing and presentation by APC followed by T cell activation and effector response. T cell response requires an additional co-stimulatory signal from co-stimulatory molecules such as CD28 for complete activation. Post-activation of effector immune cells, the activated T cells are temporarily switched off via negative regulators of T cell effector functions. This key function is required to maintain immune homeostasis. Three key negative regulators in maintaining T cell homeostasis have been identified thus far: cytotoxic T lymphocyte antigen-4 (CTLA-4), programmed cell death protein-1 (PD-1 or CD279) and programmed cell death protein ligand -1 (PDL-1) (Derks et al. 2016).

20.4.6.1 CTLA4 Inhibitors

CTLA-4 is a glycoprotein receptor expressed in both T helper ($CD4^+$) and T cytotoxic ($CD8^+$) T lymphocytes. Activation of effector T cells leads to the surface expression of CTLA-4 where it competes with CD28 and displaces it from its binding to CD80/CD86 (B7.1/B7.2) (Fig. 20.8). This leads to disruption of TCR signaling, thus attenuating the immune response and further proliferation of T cells (Sharpe and Pauken 2018). Tumors do not typically express the ligands for CTLA-4. The abrogation of the anti-tumor immune response occurs in the lymph nodes or by controlling the types and proportions of tumor-infiltrating lymphocytes (TILs). Thus CTLA-4 can be potentially targeted for improving antitumor immunity. Ipilimumab was the first FDA-approved checkpoint-blocking anticancer therapy. It is a fully human monoclonal antibody approved for advanced melanoma (Freeman et al. 2000). Tremelimumab is another anti-CTLA-4 antibody approved for malignant mesothelioma (https://www.astrazeneca.com/media-centre/press-releases/2015/

Table 20.15 List of CAR antigens (Mirzaei et al. 2017)

Antigen	Type of cancer	Domain
CD171	Recurrent/refractory neuroblastoma	CD3ζ
EGFRvIII	Glioma	CD28+CD3ζ, 4-1BB
EGFR	Gastric cancer	–
CA IX	Metastatic renal cell carcinoma	FcRγ
α-Folate receptor	Ovarian	FcRγ
HER2	Sarcoma	CD28-CD3ζ
HER2	Glioblastoma	CD28-CD3ζ
HER2	Osteosarcoma	CD28-CD3ζ
αHER2/CD3	Gastric cancer	CD28-CD3ζ
Carcinoembryonic antigen	Liver metastases	CD28-CD3ζ
IL13Rα2	Glioblastoma	CD3ζ
IL13Rα2	Glioblastoma	4-1BB, CD3ζ
HER2	Metastatic colon cancer	4-1BB, CD28, CD3ζ
GD2	Neuroblastoma	CD3ζ
GD2	Neuroblastoma	CD28, CD3ζ, OX40
ErbB2+MUC1	Breast cancer	CD28, CD3ζ SFG retroviral
HER2+CD19	Medulloblastoma	CD28+CD3ζ
Mesothelin (MSLN)	Malignant pleural mesothelioma	CD3ζ and 4-1BB
NKG2D	Breast cancer	CD28+CD3ζ
MSLN	Pancreatic cancer	CD3ζ and 4-1BB
MSLN	Malignant pleural mesothelioma	CD3ζ and 4-1BB

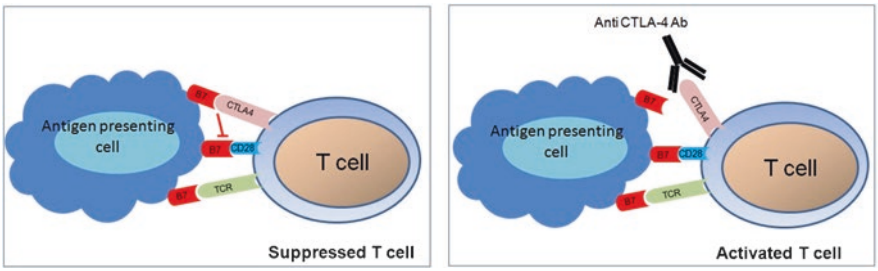


Fig. 20.8 Anti-tumor mechanism of CTLA-4 antibodies (Farkona et al. 2016)

tremelimumab-orphan-drug-designation-us-fda-malignant-mesothelioma-treatment-15042015.html#!). The exact mechanism of anti-CTLA-4 antibodies in attenuation of T cell function is ambiguous. They may possibly bind directly to effector T cells to release the check brakes or to the CTLA-4 expressed on Tregs to cause Treg cell lysis and depletion, thus limiting the infiltration of immunosuppressive Tregs into the tumor microenvironment (Tai et al. 2012).

20.4.6.2 PD1 and PDL-1 Inhibitors

Programmed cell death protein 1 (PD1) is a member of CD28 family of receptors expressed on activated T cells, T regulatory cells, B cells, NK cells and some myeloid cell populations. PD1 regulates acute and chronic inflammation, tumor proliferation and autoimmunity and is a key player in immune homeostasis. PD-1 binds to its ligands, PDL-1 (B7-H1 or CD274) or PDL-2 (B7-H2 or CD273) Okazaki and Honjo 2007; Ribas 2015. PDL1/PDL2 secretion by the tumor cells assists in tumor evasion. The binding of PD-1 on activated T cells by PDL-1 or PDL-2 results in the recruitment of phosphatases to the immune synapse which dephosphorylate the molecules involved in TCR signaling, thus abrogating the immune signaling cascade (Fig. 20.9) (Gong et al. 2018). Whilst PDL-1 is not highly expressed in non-inflamed normal tissues, many tumors have been demonstrated to express high levels of PDL-1, allowing for attenuation of the anti-tumor immune response (Ribas 2015). Both anti-PD1 and anti-PDL-1 molecules accelerate anticancer immune response. Many of the PD-1 and PDL-1 antibodies are being explored in the

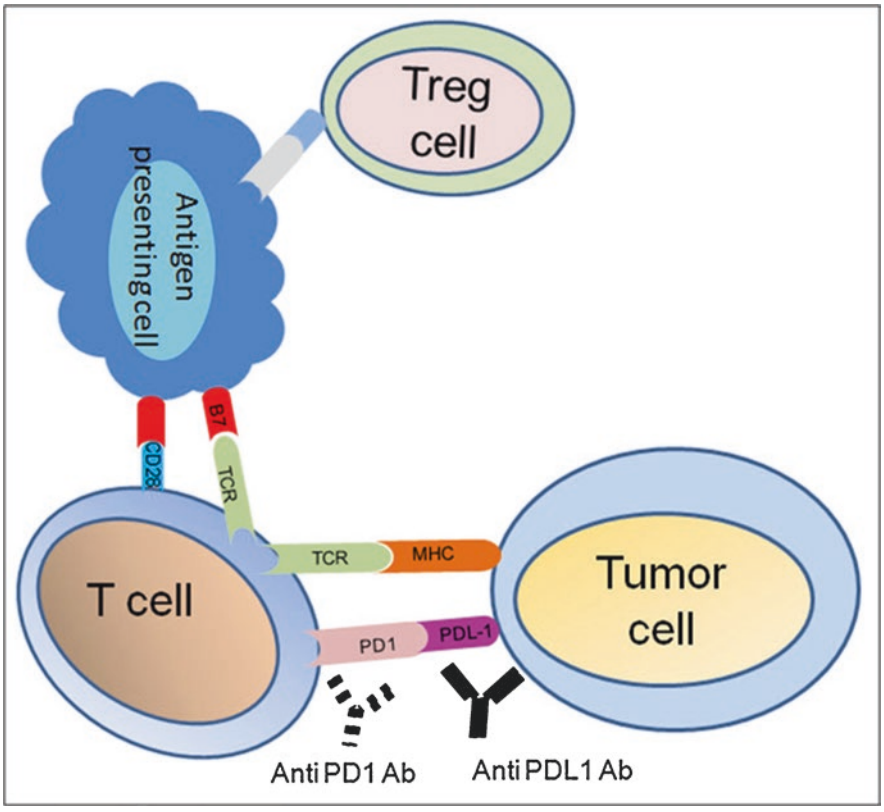


Fig. 20.9 Mechanism of PD1 and PDL1 inhibitors (Gong et al. 2018)

frontline setting, either as a single agent or in combination with chemotherapy. PD-1 inhibitors developed and now approved by the US FDA include pembrolizumab and nivolumab. Approved PDL-1 inhibitors include atezolizumab, durvalumab and avelumab (Farkona et al. 2016; Ribas 2015; Gong et al. 2018). Pembrolizumab is a human IgG4 monoclonal antibody approved as a single agent in unresectable and metastatic melanoma, having proven superior overall survival in comparison to ipilimumab. It is also approved in PDL-1-positive NSCLC after failure of platinum-doublet chemotherapy (Linch et al. 2015). Nivolumab is anti-PD1 human IgG4 monoclonal antibody approved as a single agent in unresectable and metastatic melanoma with wild-type BRAF V600, in squamous and non-squamous NSCLC after failure of platinum-doublet chemotherapy and in renal cell carcinoma after failure of prior antiangiogenic therapy (Ribas 2015). Atezolizumab is a fully humanized, engineered monoclonal antibody of IgG1 isotype against PD-L1 and is approved for urothelial carcinoma (including bladder cancer) and NSCLC. Durvalumab is an anti-PDL1 human immunoglobulin G1 kappa (IgG1κ) monoclonal antibody approved for the treatment of patients with locally advanced or metastatic urothelial carcinoma who are non-responsive to platinum-based chemotherapy. Avelumab is anti-PDL-1 human immunoglobulin G1 (IgG1) monoclonal antibody approved as second-line therapy for the treatment of advanced or metastatic urothelial carcinoma and chemotherapy-resistant metastatic Merkel cell carcinoma (Gong et al. 2018). Development of PD1/PDL1 checkpoint inhibitors in cancer treatment has witnessed an unprecedented growth in recent time. Since 2014, two anti-PD-1 and three anti-PDL-1 inhibitors have been approved, thus marking the promising treatment opportunity with this class of immunotherapy.

20.4.7 OX40 Agonists

OX40 (CD134) is a cell surface glycoprotein belonging to tumor necrosis factor (TNF) receptor super family. It is expressed on activated CD4 and CD8 T cells. Co-stimulatory signals from OX-40 aid in proliferation and survival of memory and effector T cells (Linch et al. 2015). MEDI0562 is a fully humanized OX40 monoclonal antibody, with potential immunostimulatory activity. A list of OX40 agonists undergoing clinical trials is given in Table 20.16 (https://www.onclive.com/publications/oncology-live/2017/vol-18-no-5/ox40-agonists-forge-a-path-in-combination-immunotherapy?p=2).

20.4.8 Targeting VEGF/VEGFR to Modulate Antitumor Immunity

VEGF/VEGFR signaling pathway is gaining recognition as an important regulatory pathway in cancer immunotherapy. VEGF pathway inhibitors have shown to enhance TILs (tumor-infiltrating lymphocytes) and effector T cells. They also reduce immunosuppressive Tregs, TAMs, MDSCs and mast cells and inhibit immunosuppression (Yang et al. 2018a). Increased MDSC, TAMs and mast cells are

Table 20.16 OX40 agonists in clinical trials

Inhibitor	Status
MEDI6469	Phase I/II
MEDI6383	Phase I
MEDI0562	Phase I
PF-04518600	Phase I
MOXR0916	Phase I
GSK3174998	Phase I
INCAGN01949	Phase I/II

https://www.onclive.com/publications/oncology-live/2017/vol-18-no-5/ox40-agonists-forge-a-path-in-combination-immunotherapy?p=2

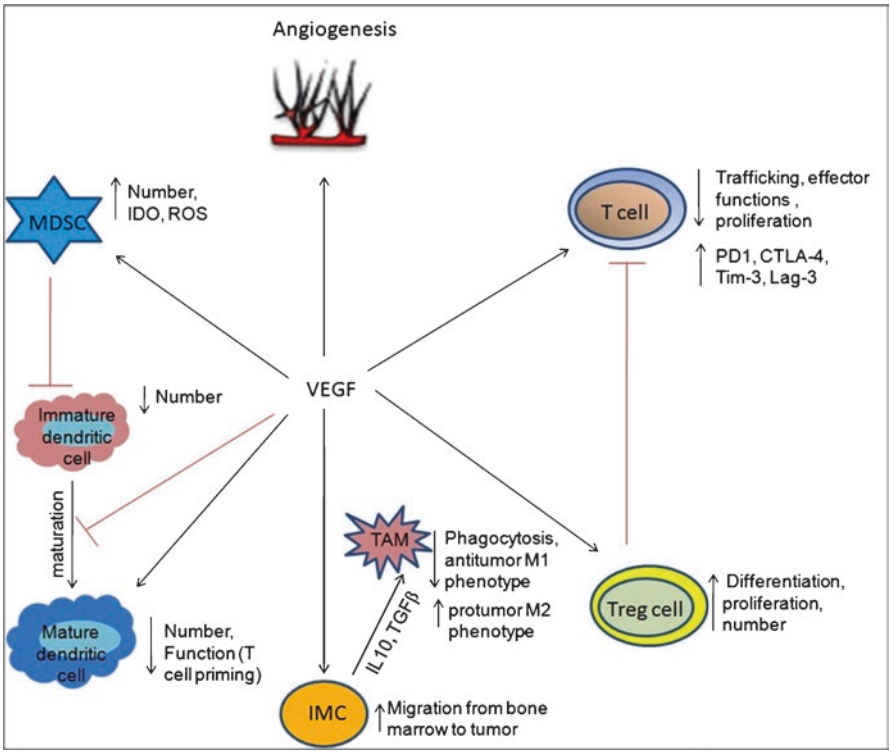


Fig. 20.10 Role of VEGF/VEGFR in modulation of immune cells (Yang et al. 2018b; Fukumura et al. 2018)

associated with resistance to kinase targeted therapies (Fig. 20.10). VEGF controls immune cell trafficking to the tumor through altered expression of adhesion molecules, intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion protein 1 (VCAM1), on endothelial cells and immune cells. Tumor-associated

endothelial cells can also augment the expression of PD-L1, which consequently binds to PD-1 expressed on T cells, thereby suppressing their anticancer activity. VEGF alone or in combination with other tumor-secreted factors (e.g. CCL2, CCL28, CXCL8, CXCL12, ANG2, PlGF and adenosine) can promote the recruitment of immunosuppressive cells (Yang et al. 2018b; Fukumura et al. 2018). Drugs targeting VEGF/VEGFR can thus be combined with other immunotherapy agents. Such combinations may be clinically synergistic through normalization of the abnormal tumor vasculature and restoration of an immune-supportive TME. VEGF inhibitors being tested in combinations with immune checkpoint blockers include bevacizumab in combination with ipilimumab, atezolizumab, nivolumab or pembrolizumab; aflibercept in combination with pembrolizumab; and TKIs sunitinib, axitinib or cabozantinib in combination with nivolumab, pembrolizumab or avelumab (clinicaltrials.gov). Angiopoietin-2 (Ang-2), an important player in angiogenesis, is another target that can be potentially combined with immune checkpoint inhibitors. Currently a humanized tri-specific biological agent BI 836880 comprising of two single variable domains blocking VEGF and Ang2 and an additional albumin module for half-life extension are in clinical trials (NCT02689505, NCT02674152) (Zirlika and Duyster 2018).

20.4.9 Targeting Tumor Metabolism to Improve Immunotherapy

The TME is a highly immunosuppressive component of any tumor-infected tissue. Tumor cell-mediated metabolic alterations of the TME make it practically unfit for tumor-infiltrating immune cells. Common features of the TME such as depleted nutrients (amino acids, glucose), low pH, high lactate levels and dysregulated immune mediators influence T cell metabolism, thereby reducing their activation and proliferation. Targeting tumor metabolism can alter a hostile immunosuppressive TME and overcome resistance to immunotherapies. Metabolic alterations in response to oncogenic factors can be exploited to alter nutrient traffic and activate antitumor immune cells to prevent cancer proliferation and spread. Various metabolic nodes can be targeted for restoration of effective immune response to tumor cells (Fig. 20.11) (Kouidhi et al. 2018; Munn and Mellor 2016). In addition to the metabolic targets visited earlier in Sect. 20.3 of this chapter, metabolic targets such as indoleamine 2,3-dioxygenase-1 (IDO1) can act as immune breaks through metabolic reprogramming and hence are being discussed under the section of immunotherapy.

20.4.9.1 IDO Inhibitors

Indoleamine 2,3-dioxygenase-1 (IDO1) is a key enzyme in tryptophan metabolism. It regulates immune cell metabolism and anti-inflammatory response through conversion of tryptophan to kynurenine. Kynurenine creates a TME inconducive to cytotoxic T cells and NK cells and potentiates activity of immunosuppressive CD4+ Treg and MDSC. High levels of IDO expression in human cancers are associated with poor disease prognosis. IDO1 promotes tumor neovascularization by

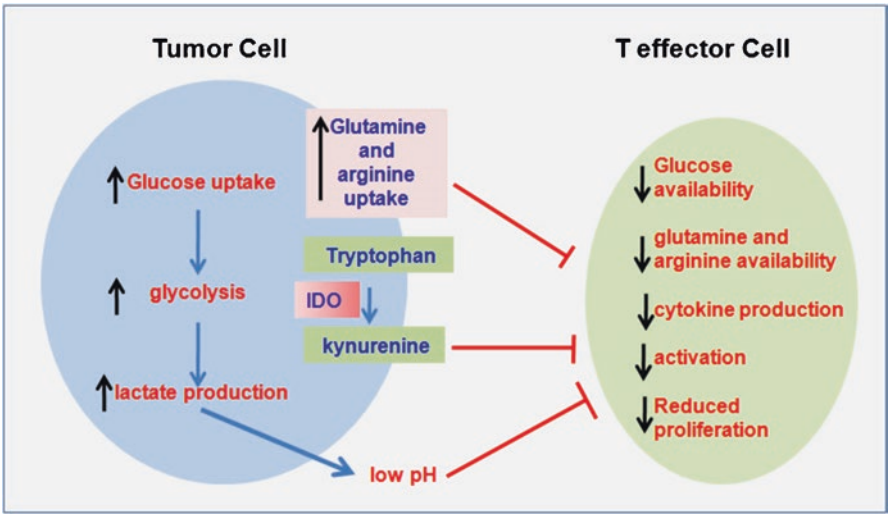


Fig. 20.11 Metabolic targeting and TME (Kouidhi et al. 2018; Munn and Mellor 2016)

Table 20.17 IDO inhibitors in clinical trials (Prendergast et al. 2017)

Inhibitor	Mechanism	Clinical status
Indoximod	Stimulates mTORC1 downstream of IDO1/TDO	Phase III
Navoximod	Catalytic inhibitor	Phase IB
Epacadostat	Catalytic inhibitor	Phase III
BMS-986205	Suicide inhibitor	Phase II
PF-06840003	Catalytic inhibitor	Phase I

regulating key cytokines IFNγ and IL6 (Prendergast et al. 2014). In vivo pharmacology and genetic studies indicate the role of IDO1 in reprogramming of TME to an inflammatory, proangiogenic and immune-tolerant TME that supports tumor growth. Preclinical studies have indicated a synergistic role of IDO inhibitors with cytotoxic or targeted chemotherapy, radiotherapy, immune checkpoint therapy and cancer vaccines (Prendergast et al. 2014; Munn and Mellor 2016). Several IDO inhibitors are under clinical trials (Table 20.17) (Prendergast et al. 2017). Few of these inhibitors in addition to IDO1 also target IDO2 and tryptophan 2,3-dioxygenase (TDO) that are involved in tryptophan catabolism. Along with IDO1, TDO can influence inflammatory modulation of TME. Thus dual IDO/TDO inhibitors can increase their efficacy and overcome resistance to IDO1-targeted therapies. Currently, three selective IDO1 inhibitors are being studied in early phase trials: epacadostat (INCB024360), navoximod and BMS-986205. Epacadostat and navoximod catalytically exhibit tryptophan competitive and non-competitive kinetic activity, respectively, whereas BMS-986205 is an irreversible IDO inhibitor (Prendergast et al. 2017).

In recent years immunotherapy has evolved as a promising cancer treatment arm and approaches cited above provide enough evidence for development of immunotherapies in the near future.

20.5 Conclusion

Changes in anticancer drug discovery through 'OMICS' guidance have yielded a favourable clinical response with both small molecules and biologics. Despite the advances, characteristic features of cancer pathology such as cellular heterogeneity continue to pose major challenges in anticancer drug development. The problem is further exacerbated due to rapid development of therapy resistance and lack of tumor-targeting drug delivery systems. Following the cracking of the human genetic code, in the past two decades, immense research has gone into identification of tumor-specific driver oncogenes that can act as drug targets. Oncogenes such as B-Raf, PIK3CA, ALK, etc., have been exploited to obtain efficacious drugs that are either approved or are in late-stage clinical trials. Some key driver oncoproteins like K-Ras are still undruggable. These targets are potential but are designated as 'yet to be drugged' targets. However that status may soon change as exemplified by the discovery of ARS1620 for K-Ras (G12C) as described under Sect. 20.2.3.2. A further challenge associated with approved cancer therapies is emergence of drug resistance. With newer drug resistance, mutations are being clinically observed with targeted therapies. We need to ask if we were wise enough to pursue these targets as well as continuously evolve in our evaluation of the biological, chemical, structural and functional aspects of the key oncotargets and their modulation through creative drug discovery approaches. In addition to the continuous exploration for druggable targets, exploiting cancer metabolism to create TME changes that favour antitumor immune response and tumor metabolic deprivation will help to re-establish healthy tissue homeostasis. This in turn may overcome the hurdles associated with containment of the tumor. Interplay of oncogenic signaling especially through growth factor modulated pathways, immune surveillance, altered tumor metabolism and neo-angiogenesis can be exploited to obtain novel therapeutics. Novel emerging targets in the area of cancer signaling, metabolism and immunomodulation as standalone or combination therapy are thus poised to offer new standards of care.

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